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INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR
UNIVERSIDADE DO PORTO

IN VITRO SCREENING OF THE ANTICANCER ACTIVITY OF MARINE AND SOIL-DERIVED FUNGI EXTRACTS AND COMPOUNDS USED ALONE AND COMBINED WITH DOXORUBICIN:

Evaluation of the anticancer properties of fungi extracts and compounds alone and in combination with doxorubicin in lung cancer cells

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DISSERTAÇÃO DE MESTRADO APRESENTADA

AO INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

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CIÊNCIAS DO MAR – RECURSOS MARINHOS

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O NOVO NORTE
PROGRAMA OPERACIONAL
REGIONAL DO NORTE

Abstract

Cancer is a worldwide cause of concern. The incidence, prevalence and death rates of this disease have not yet been considerably attenuated over the years. Novel and innovative treatments are emerging as a result of the increasing knowledge on the biology of cancer. Cancer cells seem to adapt and evolve new evasive pathways along with the progression of cancer therapy. Multi-drug resistance is a characteristic acquired by certain types of cancer, such has lung cancer, that severely decreases the efficiency of chemotherapy. One of the approaches explored to mitigate this issue is the implementation of combined drug therapy. Natural products have been long-term “allies” of mankind in the treatment of disease. In the last decades the marine environment has gained interest as a plentiful source of natural products with multiple biotechnological applications. Cancer treatment is one of the fields that is benefiting from marine-derived products. Indeed, the search for new drugs with unique mechanisms of action is of clear interest, and the marine environment, which is still largely untapped, may provide answers to many of the pharmaceutical requests of today.

In our study the *in vitro* anticancer activity of crude ethyl acetate extracts of marine-derived fungi *Neosartorya tsunodae* KUFC 9213 (**E1**) and *Neosartorya laciniosa* KUFC 7896 (**E2**) and soil fungus *Neosartorya fischeri* KUFC 6344 (**E3**) were tested on a panel of seven human cancer cell lines (HepG2, HCT116, HT29, A549, A375, MCF-7 and U251). The *in vitro* anticancer activity of these extracts was assessed by several forms, in particular, short-term proliferative activity was assessed by an MTT assay, DNA damage was evaluated by an alkaline comet assay, effect on long-term proliferative activity was evaluated by a clonogenic assay and cell death was measured by quantification of cell exhibiting nuclear condensation and observation of morphological alterations. Extract **E2** demonstrated a clear *in vitro* anticancer effect by decreasing the clonogenic potential and increasing the induction of nuclear condensation in HCT116, A375, MCF7 and HT29 cells. Extract **E3** demonstrated similar effects in MCF7 and HCT116 cells. The induction of DNA damage was observed in some cell lines. These results suggest that extracts **E2** and **E3** possess an *in vitro* anticancer effect by inhibiting proliferation and inducing cell death in human colon carcinoma, breast adenocarcinoma and malignant melanoma cells, validating the interest for the identification of compounds isolated from these extracts in further studies.

The second phase of this work intended to evaluate the enhancement of the *in vitro* anticancer activity of doxorubicin while in combination with the crude ethyl extracts of seven marine and soil-derived fungi, namely, *Neosartorya tsunodae* KUFC 9213 (**E1**), *Neosartorya laciniosa* KUFC 7896 (**E2**), *Neosartorya fischeri* KUFC 6344 (**E3**), *Aspergillus similanensis* KUFA 0013 (**E4**), *Neosartorya paulistensis* KUFC 7894 (**E5**), *Talaromyces trachsypermus* KUFC 0021 (**E6**) and *Neosartorya siamensis* KUFA 0017 (**E7**) in the same panel of seven cancer cell lines. Eight compounds isolated from *Neosartorya siamensis* KUFA 0017 (**E7**)**,** namely, 2,4-dihydroxy-3-methylacetophenon (**C1**), nortryptoquivaline (**C2**), chevalone C (**C3**), tryptoquivaline H (**C4**), fiscalin A (**C5**), *epi*-fiscalin C (**C6**), *epi*-neofiscalin A (**C7**) and *epi*-fiscalin A (**C8**) were tested in combination with doxorubicin and cell viability was assessed in A549 lung cancer cell line. Extracts **E1**, **E2** and **E7** in combination with doxorubicin demonstrated a significantly enhanced effect in A549 cells, in comparison to the sole use of doxorubicin. A significant increase in DNA damage and nuclear condensation was also observed. Due to the effect demonstrated by extract **E7** in combination with doxorubicin, eight compounds of this extract were tested in different combinatorial regimens in A549 cell line. All the isolated compounds (except **C1)** in combination with doxorubicin potentiate the cytotoxic effect of the latter drug. Compounds **C2**, **C5** and **C7** in combination with doxorubicin demonstrated a greater effect in the decrease of cell viability when compared with extract **E7** in combination with doxorubicin. Our data demonstrated the potential in enhancing (at least *in vitro*) the anticancer effect of doxorubicin by the use of marine-derived fungi extracts and compounds.

Resumo

O cancro é uma preocupação que atinge a escala mundial. A incidência, prevalência e taxa de mortalidade desta doença não tem atenuado ao longo dos anos. Alguns tratamentos inovadores têm emergido como resultado do crescente conhecimento da biologia do cancro. Contudo, as células cancerígenas adaptam-se e desenvolvem novos mecanismos de evasão ao acompanhar a progressão do tratamento. A resistência a múltiplas drogas é uma característica verifica em certos tipos de cancro, nomeadamente o cancro do pulmão, e que diminui drasticamente a eficiência e resposta à quimioterapia. Uma das abordagens adoptadas para a mitigação deste problema é a implementação de terapias com combinação de drogas. Os produtos naturais são "aliados" ancestrais da humanidade no tratamento de doenças. Nas últimas décadas, o ambiente marinho tem sido alvo de grande interesse como uma fonte abundante de produtos naturais com múltiplas aplicações biotecnológicas. Inclusivamente, o tratamento do cancro tem sido uma das áreas beneficiadas por novos produtos de origem marinha. Torna-se assim evidente que a procura de novas drogas com mecanismos de acção únicos é de extrema relevância, e que o ambiente marinho, ainda vastamente inexplorado, pode providenciar respostas às necessidades farmacêuticas actuais.

Neste estudo, a actividade anticarcinogénica *in vitro* de extractos de acetato de etilo de fungos de origem marinha, nomeadamente, *Neosartorya tsunodae* KUFC 9213 (**E1**) e *Neosartorya laciniosa* KUFC 7896 (**E7**) e do fungo de solo *Neosartorya fischeri* KUFC 6344 (**E2**), foi avaliada num painel de sete linhas celulares (HepG2, HCT116, HT29, A549, A375, MCF-7 e U251). A actividade anticarcinogénica *in vitro* destes extractos foi avaliada de várias formas, nomeadamente, através da avaliação de inibição de proliferação celular a curto prazo pelo ensaio de MTT, da observação de indução de danos no DNA através do ensaio cometa, da inibição da proliferação celular a longo prazo, através do ensaio clonogénico, e da avaliação da indução de morte celular pela quantificação de condensação nuclear e por observação de alterações morfológicas. O extracto **E7** evidenciou um claro efeito anticarcinogénico *in vitro* ao diminuir o potencial clonogénico das células, bem como ao aumentar a indução de condensação nuclear em células HCT116, A375, MCF7 e HT29. O extracto **E2** demonstrou efeitos semelhantes em células MCF7 e HCT116. A indução de danos no DNA foi observada em algumas linhas celulares. Estes resultados sugerem que os extractos **E7** e **E2** possuem actividade anticarcinogénica *in vitro* ao inibir a proliferação e ao induzir a morte celular em células de carcinoma do có-

lon, adenocarcinoma da mama e melanoma maligno, validando assim o interesse na identificação de compostos isolados destes extractos em estudos futuros.

A segunda fase deste estudo pretendeu avaliar a potenciação do efeito anticarcinogénico *in vitro* da doxorrubicina quando combinada com os extractos de acetato de etilo obtidos de sete fungos marinho e um fungo de solo, nomeadamente, *Neosartorya tsunodae* KUFC 9213 (**E1**), *Neosartorya laciniosa* KUFC 7896 (**E2**), *Neosartorya fischeri* KUFC 6344 (**E3**), *Aspergillus similanensis* KUFA 0013 (**E4**), *Neosartorya paulistensis* KUFC 7894 (**E5**), *Talaromyces trachsypermus* KUFC 0021 (**E6**) e *Neosartorya siamensis* KUFA 0017 (**E7**) no mesmo painel de sete linhas celulares. Oito compostos isolados do fungo *Neosartorya siamensis* (**E7**), nomeadamente, 2,4-dihydroxy-3-methylacetophenon (**C1**), nortryptoquivaline (**C2**), chevalone C (**C3**), tryptoquivaline H (**C4**), fiscalin A (**C5**), *epi*-fiscalin C (**C6**), *epi*-neofiscalin A (**C7**) e *epi*-fiscalin A (**C8**) foram testados em combinação com doxorubicina na linha celular de pulmão A549. Os extractos **E1**, **E2** e **E7**, quando combinados com doxorrubicina, demonstraram um efeito significativamente mais potente em células A549, em comparação com o uso exclusivo da doxorrubicina. Foi também observado um aumento significativo de indução de danos no DNA e de condensação nuclear. Face à magnitude do efeito do extracto **E7** combinado com doxorrubicina, foram testados oito compostos isolados deste extracto em vários emparelhamentos combinatórios com doxorrubicina, na linha celular A549. Quando combinados com aquele fármaco, todos os compostos isolados, à excepção do composto **C1**, demonstraram um maior decréscimo de viabilidade celular em comparação ao uso de extracto **E7** com doxorrubicina. Estes resultados demonstraram, de forma inequívoca, a potencialidade de se utilizarem extratos e compostos de fungos de origem marinha para potenciar (pelo menos *in vitro*) o efeito anticarcinogénico da doxorrubicina.

Index

Abbreviations

A375 – Human Malignant Melanoma Cell Line

A549 – Human Non-small Lung Cancer Cell Line

BEL-7402 – Human Hepatocellular Carcinoma Cell Line

DAPI – 4',6-diamidino-2-phenylindole

DMEM – Dulbecco's Modified Eagle Medium

DMSO – Dimethyl sulfoxide

DNA – Deoxyribonucleic acid

Dox – Doxorubicin

E1 – *Neosartorya tsunodae* KUFC 9213

E2 – *Neosartorya laciniosa* KUFC 7896

E3 – *Neosartorya fischeri* KUFC 6344

E4 – *Aspergillus similanensis* KUFA 0013

E5 – *Neosartorya paulistensis* KUFC 7894

E6 – *Talaromyces trachsypermus* KUFC 0021

E7 – *Neosartorya siamensis* KUFA 0017

EDTA – Ethylenediaminetetraacetic acid

EGFR – Epidermal Growth Factor Receptor

FBS – Fetal Bovine Serum

FDA – Food and Drug Administration

GST – Glutathione S-transferase

GSTM1 – Glutathione S-transferase Mu 1 Gene

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HCT116 – Human Colon Carcinoma Cell Line

HepG2 – Human Hepatocellular Carcinoma Cell Line

HL-60 – Human Promyelocytic Leukemia Cell Line

HRAS – Harvey Rat Sarcoma Viral Oncogene Homolog

Hs683 – Human Oligodendroglioma Cell Line

HT29 – Human Caucasian Colon Adenocarcinoma Grade II Cell Line

IC_{50} – Half Maximal Inhibitory Concentration

KRAS – Kirsten Rat Sarcoma Viral Oncogene Homolog

LMP – Low Melting Point Agarose

MCF-7 – Human Breast Adenocarcinoma Cell Line

MDR1 – Multidrug Resistence Protein 1 Gene

MEM – Minimum Essential Medium Eagle

MOLT4 – Human Acute Lymphoblastic Leukemia Cell Line

MRP – Multiple Drug Resistance

MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NCI-H460 – Human Non-small Lung Cancer Cell Line

NSCLC – Non-small Cell Lung Cancer

NMP – Normal Melting Point Agarose

P388 – Murine Lymphocytic Leukemia Cell Line

PBS - Phosphate Buffered Saline

PFA – Paraformaldehyde

P-gp – P-glycoprotein

RPMI – Roswell Park Memorial Institute Medium

SK-MEL-28 – Human Malignant Melanoma Cell Line

U251 – Human Glioblastoma Astrocytoma Cell Line

U373 – Human Glioblastoma Cell Line

SGC-7901 – Human Gastric Cancer Cell Lines

TP53 – Tumor Protein p53 Gene

Preface

This work was developed in the course of the MARBIOTECH project in the Interdisciplinary Centre of Marine and Environmental Research (CIIMAR), of the University of Porto, which intends to obtain and evaluate different applications for the bioactivity of marine-derived products. With this work, our contribution to this project was by assessing the *in vitro* anticancer activity of marine and soil-derived fungi extracts and compounds.

In particular, the aim of this study was to assess the *in vitro* anticancer activity — by evaluating the anti-proliferative activity, clonogenic potential, DNA damage and induction of nuclear condensation — of marine and soil-derived fungi extracts and compounds. Additionally the potentiation of the *in vitro* anticancer activity of doxorubicin was evaluated by using combinatorial regimens of extracts and compounds in combination with doxorubicin.

This thesis is presented over four chapters. Chapter 1 contains an introduction to cancer where epidemiologic data, cellular and molecular traits (with special focus in the biology of lung cancer), therapeutic procedures for lung cancer are approached. Aspects of natural products, pharmaceutical applications of marine-derived drugs and a view over drug screening are equally presented. Chapter 2 includes an original manuscript submitted to an international peer-reviewed journal, while Chapter 3 illustrates an original manuscript to be submitted to an international peer-reviewed journal. Chapter 4 presents the global conclusions and introduces key future perspectives. The appendix includes a comprehensive presentation of the experimental protocols used over this thesis.

List of publications

This master's thesis allowed the preparation and publication of the following works:

Original manuscripts submitted to international peer-reviewed journals

Ramos A. A.*, **Castro-Carvalho B.***, Prata-Sena M., Dethoup T., Buttachon S., Kijjoa A. & Rocha E. (2014) Extracts from *Neosartorya* (fungi) species exhibit anti-proliferative activity with cell death induction in colon, breast and skin cancer cell lines.

Ramos A.A.*, Prata-Sena M.*, **Castro-Carvalho B.**, Dethoup T., Buttachon S., Kijjoa A. & Rocha E. (2014) Testing the potential of four marine-derived fungi extracts as anti-proliferative and cell death-inducing agents in seven human cancer cell lines.

Original manuscripts to be submitted to peer-review international journals

Castro-Carvalho B., Ramos A. A., Prata-Sena M., Dethoup T., Buttachon S., Kijjoa A. & Rocha E. (2014) Marine-derived fungi extracts and isolated compounds enhance the anticancer activity of doxorubicin in non-small cell lung cancer cells. *To be submitted.*

Prata-Sena M., Ramos A., **Castro-Carvalho B.**, Dethoup T., Buttachon S., Kijjoa A. & Rocha E. (2014) Cytotoxic activity of isolated compounds from marine-derived fungi *Neosartorya siamensis* in human cancer cells. *To be submitted.*

Abstracts published in international peer-reviewed journals

Castro-Carvalho, B., Ramos, A., Prata-Sena, M., Dethoup, T., Buttachon, S., Kijjoa, A., & Rocha, E. (2014). Extracts from the marine fungus *Neosartorya tsunodae* and the soil fungus *Neosartorya fischeri* exhibit anti-proliferative and pro-apoptotic effects in human cancer cell lines. *Planta Medica, 80*(16), P1N32. doi: 10.1055/s-0034-1394622

Ramos, A., Moreira, M., **Castro-Carvalho, B.**, Prata-Sena, M., Dethoup, T., Buttachon, S., Kijjoa, A., Rocha, E. (2014). Marine-derived fungi extracts increase doxorubicin's cytotoxic effect in lung cancer cells. *Planta Medica, 80*(16), P1N25. doi: 10.1055/s-0034-1394615

Prata-Sena, M., Ramos, A., **Castro-Carvalho, B.**, Dethoup, T., Buttachon, S., Kijjoa, A., & Rocha, E. (2014). Anti-proliferative and pro-apoptotic activities of two marine sponge-derived fungi extracts in HepG2, HCT116 and A375 cancer cell lines. *Planta Medica, 80*(16), P1N3. doi: 10.1055/s-0034-1394594

Ramos, A., Malhão, F., Ferreira, A., Alves, Â., **Castro-Carvalho, B.**, Prata-Sena, M., Gargiulo, D., Dethoup, T., Buttachon, S., Lobo-da-Cunha, A., Kijjoa, A., Rocha, E. (2014) Marine and soil fungi extracts with anti-proliferative activity induce morphological alterations in breast cancer cells. *Microscopy and Microanalysis (in press)*

Communications

Two poster presentations at the 62nd International Congress and Annual Meeting of the Society for Medicinal Plant and Natural Product Research – GA2014, between the 31st of August and 4th of September 2014, in Guimarães.

CHAPTER 1

GENERAL INTRODUCTION

1. Introduction

1.1. Perspectives on human health and natural products

The use of crude or more refined products from nature in order to acquire health benefits is an ancient human habit, however, the consumption of many of these products has been scientifically proven to offer chemoprevention/protection for several human diseases. Cancer is frequently embraced by society as a modern disease due to its increasing prevalence among human populations; in fact, cancer has accompanied human civilizations throughout time. Some of the earliest records of cancer as an illness dates back to 3000 B.C. in Egypt, where the tumors were surgically removed and the disease was reported as not possessing a definitive treatment (Sudhakar, 2009). The use of natural products with the intent to treat or prevent, dates back from the 12th century among Asian populations (Cai, Luo, et al., 2004). Plants were the initial target for a deeper search of bioactive compounds, due to their abundance, easy maintenance and propagation, and by the already demonstrated benefits among populations who actively consumed certain plant species as a cultural habit. The effective use of plants as a medicinal tool relies upon the plant's production of secondary metabolites, which serve not only as the plant's self-defense mechanism, but also have the propensity to promote health improvements when included in the human diet (Briskin, 2000). Several of these products may possess molecules with several biological activities, such as anti-oxidant, anti-inflammatory and anti-proliferative potential, which could contribute for cancer prevention and treatment (Rathee, Holt, et al., 2009; Thomson and Ali, 2003). Consequently, the search for bioactive compounds in nature has been widely validated by the number of drugs integrating the pharmaceutical industry. Many of these drugs were discovered by inspiration or origin in nature's portfolio, which is particularly true for cancer treatment: from 1983 to 1994 about 60% of FDA approved drugs had its origin in nature, either by plants, microorganisms or marine organisms (Newman, Cragg, et al., 2003). Since the 1960s, the effort to obtain drugs was focused towards the ocean, and by the mid-1980s around 2500 new metabolites were reported (Cragg and Newman, 2013). This came to prove the enormous potential in the search of novel bioactive compounds from marine-derived sources.

Considering the ocean as a rather generous source of products with high potential for medicinal application which could very well break even with land-derived products not only due its dimension, but also because marine organisms have faced millions of years of evolution and selective pressure. Therefore, they must possess diverse and competent chemical machinery that enables their survival in the harsh and constantly changing con-

ditions of the marine environment (Boopathy and Kathiresan, 2010). The chemical portfolio of marine organisms may find its most seamless and complex functionality amongst symbiotic organisms, since symbiotic organisms have been found to interact and aid each other using unique chemical output. Such products hold great potential, in particular due to the possibility of discovering novel chemical structures with biotechnological applicability, namely for cancer treatment (Webster and Taylor, 2012).

Chemotherapeutic agents for cancer therapy have been in clinical use for about half a century, and although some have proven efficiency in some type of cancers, in others, such as lung cancer, chemotherapy has not yet been able to overcome its puzzling biology (Shanker, Willcutts, et al., 2010). The efficiency of a drug relies upon its ability to induce cytotoxicity or/and cytostatic effect among the tumor cells (Hickman, 1992; Villasana, Ochoa, et al., 2010). Highly specific drugs are especially desirable, considering that although it is critical to damage and limit cancer cells, it is also important to avoid the same effects on normal cells. A cytotoxic effect is often achieved by activating a cell death mechanism. This event can result from drug induction of apoptotic molecular pathways, which are often mutated in cancer cells (Greenblatt, Bennett, et al., 1994). The importance of drug-induced apoptosis is easily demonstrated, if one considers that around 80% of the currently used chemotherapy drugs function by activating a pro-apoptotic mechanism (Balde, Andolfi, et al., 2010).

Most types of cancer become drug resistant during the administration of chemotherapeutic agents to patients (Gottesman, 2002). Lung cancer is a classic example of a cancer with ample drug resistance mechanisms; this is in fact portrayed in the high prevalence and poor treatment prognosis, which is translated into a high mortality rate (Jemal, Bray, et al., 2011; Shanker, Willcutts, et al., 2010). One of the approaches that is commonly enforced to minimize drug resistance is the implementation of drug combination therapies, in order to explore multi-target mechanisms within the cancer cell (Belani, Choy, et al., 2005).

Finding new natural bioactive drugs requires the evaluation of the drug by means of a screening, where several characteristics of these new drugs are evaluated (Shoemaker, Scudiero, et al., 2002). Once the drug's bioactivity is assessed, illations may be made as to whether the drug can be considered as a valid candidate to be combined with other drugs, thus hopefully resulting in the overall enhancement of bioactivity in relation to the use of both drugs alone (Zhang, Teruya, et al., 2013).

1.2. Cancer demographics

The burden of cancer is a matter that involves personal, social and governmental spheres. The concept of cancer has reached our daily living as a subject of present and future concern, and it certainly represents the face of an unexpected, sudden and a painful death. Environmental factors, aging, life-style and familiar history are major contributions towards the incidence, prevalence and mortality of cancer. Cancer is a global leading cause of mortality, being the first cause of fatal diseases in more developed countries, and the second in the less developed countries (Jemal, Bray, et al., 2011).

1.2.1. Current trends in cancer incidence

A global estimate in 2012, estimated that 8.2 million people died of cancer, accompanied by the appearance of 14.1 million new cases (Ferlay, Soerjomataram, et al., 2014). In contrast, in 2008 there was an estimation of 7.6 million deaths (64% in developing countries), and 12.7 million new cases (56% in developing countries) (Jemal, Bray, et al., 2011). Even more markedly, estimates of the year 2000 suggested that 10.1 million of new cases and 6.2 million deaths had occurred that very year; this represented an increase of 20% in both parameters, in comparison with the estimations for the year of 1990 (Parkin, 2001). An interpretative analysis of these two periods must consider the high global population that occurred between these years. The lack of proper and well implemented diagnosis methods alongside the patient's difficulty to access efficient treatment may explain these high mortality rates in underdeveloped regions, even though the incidence of the disease is less prevalent (Jemal, Bray, et al., 2011). Projections for 2020 estimate a total of 15.4 million new cases and 10.1 million deaths, and by 2050, an estimate of 23.8 million new cases and 16 million deaths related to cancer (Parkin, 2001).

1.2.2. Insights in lung cancer incidence

On a global scale, lung cancer is the most common type of cancer. Lung cancer has consistently increased every year, and represents 12.3% of all cancers with a total of 52% of these cases accounting for developed countries (Parkin, Bray, et al., 2001). In terms of gender, males are the most affected, accounting for 75% of all cases. In fact, this is the most recurrent type of cancer for the gender, and the third for females (data correspond-

ent to the year 2000) (Parkin, 2001; Parkin, Bray, et al., 2001). Smoking habits are the predominant risk factor that dictates the possibility to develop the disease, in fact, 80% of lung cancers are recurrent in men and 50% in women with smoking habits (Jemal, Center, et al., 2010; Parkin, 2001). The increase of an individual's smoking habits (e.g. passive smoking, amount of smoke inhaled and early age initiation) positively increases the probability to manifest the disease. Countries with a long lasting cultural habit of smoking are now more affected by this disease, possibly as a result of more exposure time and quantitative contact with tobacco associated carcinogenic agents (Parkin et al., 2001). Familiar history, prior respiratory diseases and exposure to environmental pollutants, such as organic chemicals, radiation, asbestos, metals, chronic dust exposure, coal smoke, and other forms of air pollutants are also relevant factors for the development of lung cancer (Brenner, McLaughlin, et al., 2011; Spitz, Hong, et al., 2007). The individual's gene pool is quite relevant, since 17% of lung cancer cases are related with homozygous deletion on *GSTM1* locus, a member of glutathione S-transferases (GST) family that act as detoxifier enzymes that inhibit the formation of DNA adducts, such as smoking-related DNA adducts in lung tissues (McWilliams, Sanderson, et al., 1995).

Lung cancer histology characterizes the condition in two variants, i.e. the non-small cell lung and the small cell lung cancers. The first morphological type originates from bronchial epithelial-cell precursors and comprises large cell carcinoma, adenocarcinoma, squamous cell carcinoma and bronchioalveolar cell carcinoma, all of which account to 85% of lung cancer cases. Small cell lung cancer arises from neuroendocrine cell precursors (Marshall and Christiani, 2013; Ramalingam, Owonikoko, et al., 2011).

1.3. Biology of cancer

Carcinogenesis is not simply defined as a random growth process of an undifferentiated mass of cells. In fact, carcinogenic cells dominate and quite efficiently alter the firmly established cell cycle pathway associated to normal cells; cancer cells manipulate their surrounding microenvironment in their own advantage and ultimately avoid their host's self-defense mechanisms. These cells proclaim their own space and nutrients, as opposing to normal cells. Cancer cells are relentless in their progression and are self-invaders that control their own course (DeBerardinis, Lum, et al., 2008).

In 2011, and despite the histological origin and functional differences among the different cancer cell types, common hallmarks were attributed to cancer: sustaining proliferative

signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, activating invasion and metastasis, deregulating cellular energetics, avoiding immune destruction, genome instability and mutation and tumor-promoting inflammation. These biological characteristics conjointly promote the tumorigenic and carcinogenic process (Hanahan and Weinberg, 2011).

1.3.1. Biology of lung cancer

The detection and identification of lung cancer has made great advances through molecular and genetic identification tools (Cortes-Funes, 2002). Consequently, a more complex and elucidated mapping of this disease's biology is now known, in comparison to an entirely morphologic based analysis. Divisions based exclusively on histological criteria disregard most of the crucial molecular traits of the disease. Moreover, the discrepancy which is often observed in the efficiency of treatment amongst patients presenting the same histological cancer type is a call for the further exploration of vital information which may be found beyond morphology (Manegold, 2001; Ramalihngman, Owonikoko, et al., 2011).

At the moment, it is possible to establish a net of genetically identical traits in normal lung cells that, if present, may increase the risk of evolution towards malignancy. A region in the chromosome 15 contains the nicotinic acetylcholine receptor genes, which are thought to be involved in tobacco smoking dependence, and therefore implicated in the lung cancer mechanism (Thorgeirsson, Geller, et al., 2008). Some of the most common molecular abnormalities in lung cancer are mutations in *EGFR*, *TP53* and *KRAS* genes (Marshall and Christiani, 2013). The epidermal growth factor receptor (*EGFR)* belongs to a family of cell-surface receptors, with the function of activating cellular mechanisms mediated through extra-cellular factors. These factors could ultimately lead to the regulation of such functions, as for example apoptosis and angiogenesis (Krause and Van Etten, 2005). The *EGFR* gene is mutated in a considerable percentage of NSCLC carriers (e.g., 40% of East Asian carriers), which motivated drug trials using EGFR inhibitors, which lead to the conclusion that carcinogenic cells promptly acquire resistance to these inhibitors (Kobayashi, Boggon et al., 2005).

Transcriptor factor p53 (*TP53*) is a central tumor suppressor gene. It acts in cell cycle arrest, allowing the evaluation of internal damages (e.g. DNA damage) which will determine if the cell proceeds further in the cell cycle or if it is forwarded to initiate cell death (Li, Kon, et al., 2012). *TP53* gene is frequently mutated in lung cancer. The prevalence of

this mutation is dependent on the histological type of cancer. In one case study, it was found to be more frequent in cell squamous carcinoma (35%) when compared with adenocarcinoma (20%), yet the overall expression of this gene along the chain of the tumorigenesis events remains in some points poorly comprehended (Gao, Mady, et al., 2003).

The *RAS* gene family is composed by three proto-oncogenes (*KRAS, HRAS,* or *NRAS).* These genes have an active role in the maintenance of the proliferation, apoptosis, metastasis and immune response (Pylayeva-Gupta, Grabocka, et al., 2011). Mutations in *KRAS* and *EGFR* are rarely found simultaneously, suggesting a similar activity in lung cancer carcinogenesis (Gazdar, Shigematsu, et al., 2004). These molecular targets, as well as several others, must not be overlooked and new strategies to take advantage of cellular fragilities must continue to be explored.

1.4. Cancer therapeutics

Cancer research and therapy has been approached by multifactorial targets. The aim is to interact with the multi-altered pathways present in cancer cells, e.g. by inducing programmed cell death, arresting cell cycle, inhibiting angiogenesis and mitigate metastatic behaviors with specific therapeutics. Most importantly, it is preferable that these therapeutical approaches demonstrate selectivity towards cancer cells, thus sparing normal, functional cells (Luo, Solimini, et al., 2009).

The use of cancer therapeutics is a matter that must be planned with the most accurate and detailed diagnosis. Factors such as cancer histology, tumor phase stage and patient age must be considered when choosing the ideal treatment either through chemotherapy, radiotherapy, surgery intervention or a combination of these (Ramalingam, Owonikoko, et al., 2011).

1.4.1. Lung cancer treatment

Lung cancer demonstrates the highest rate of mortality and incidence in comparison with other cancers. Any decrease in these rates is mostly attributed to smoking cessation programs rather than currently used chemo or radiotherapeutic programs. Lung cancer carriers, namely presenting non-small cell lung cancer (NSCLC), have an average lifespan of 12 months from the initial diagnosis and a 5-year survival rate of 5-10% with

the available therapy offering no more than palliative care (Cortes-Funes, 2002; Spiro and Porter, 2002). Poorly implemented screening methods (mainly by a chest X-ray) are not sufficient to detect early pre-symptomatic stages of the disease, thus reducing the cases where patients are allowed to receive a treatment at an early stage where the disease does not yet represent a total life threat (Ramalingam, Owonikoko, et al., 2011). Chemotherapeutic agents are costly and the toxicity to the patient's organism implicates a severe decrease in quality of life (Ramalingam, Owonikoko, et al., 2011). These factors require mitigation of some kind. Finding new drugs and implementing drug combinations can significantly lower the required drug dosage, decrease side effects and ultimately improve the patient's life quality.

There are four stages of lung cancer, some of these stages are subdivided according to the extent of the lesions (Table 1). Early stages of NSCLC are considered to be stage I, stage II and certain cases of the subtype IIIA. These early stages of the disease account for approximately 30% of NSCLC carriers and surgical resection of all or part of the lung remains as the most common intervention. When a locally advanced NSCLC considered as surgical unresectable, medical treatment relies solely on chemotherapy or/and radiotherapy. When applying a combination of these treatments, with changes in dose, time exposure, and the used chemicals, survival rates seem to improve significantly in spite of the high toxic intake (Ramalingam, Owonikoko, et al., 2011). The standard procedure for the mentioned stage of the disease varies in two regimens of treatment. The first, consisting of a full dose of cisplatin and etoposide and radiotherapy, demonstrating notable side-effects to patients, but quite efficiently eliminates small clusters of metastization (Albain, 2002); and the second with carboplatin and paclitaxel, in a dose capable of reducing tumor resistance to radiotherapy, followed by an intense dose of the combination (carboplatin and paclitaxel), presenting a better physical acceptance and allowing to cope with higher doses of radiotherapy (Belani, Choy, et al., 2005). Several improvements to current therapy are under test phase (Vokes, Senan, et al., 2009), and the most promising results are expected to arise when both radiotherapy and chemotherapy fields are equally explored and developed.

Platinum combined drugs (e.g. carboplatin and cisplatin) are mostly indicated for advanced stages of the disease (stage IV). High neurotoxicity of these type of drugs has held back a wider use in other stages of the disease, thus favoring the path of combination with platinum-free drugs (Manegold, 2001). The future of NSCLC therapeutics requires tumor screening in terms of genetic characteristics and development of a more personalized treatment (Ramalingam, Owonikoko, et al., 2011). A deeper analysis of the

individual's clinical and molecular status seems suitable to a more focused and resolute fight against this illness.

Doxorubicin is an anthracycline antibiotic derived from the actinobacterium *Streptomyces peucetius*. It was discovered in the seventies and is a hydroxylated form of daunorubicin. Doxorubicin is used in the treatment of a number of cancers, including lung cancer, and acts by interacting with the enzyme topoisomerase IIα, which leads to the accumulation of DNA breaks and ultimately to cell death (Cortes-Funes and Coronado, 2007; Zunino and Capranico, 1990). Myelosuppression and cardiomyopathy the most common side effects of the use of doxorubicin and others anthracyclines (Cortes-Funes and Coronado, 2007). Their use in combination with other drugs is frequent practice to minimize side effects, doxorubicin is extensively used in combination with etoposide and cyclophosphamide in the treatment of small-cell lung cancer (Cortes-Funes and Coronado, 2007).

Table 1 – The stages of lung cancer. Adapted from (LungCancer.org, 2014).

Cancer stage	Cancer sub-stage	Cancer localization
Stage I	n.d	Cancer exclusively located in the lungs
Stage II	IIA; IIB	Cancer in the lungs and nearby lymph nodes
Stage III	IIIA;	Cancer spread to lymph nodes but in the same side of his origin
	IIIB	Cancer spread to lymph nodes but to a different side from its origin
Stage IV	n.d	Cancer has spread to both lungs, and other organs.

n.d – not defined

1.4.2. Drug resistance

Drug resistance is a feature possessed by several types of cancer. Drug resistance represents one of the main reasons for the long-term inefficacy of chemotherapeutic agents. While chemotherapeutic agents may inflict a significant cytotoxicity as a treatment is initiated, chemo-sensitive cells in the tumor are reduced, however, chemo-resistant

cells are positively selected. Subsequently, chemotherapeutic agents will no longer impact the tumor cells to the same extent as the initial treatment (Persidis, 1999).

Several mechanisms have been related with cancer drug resistance, for example, overexpression of drug transporters (e.g. P-glycoprotein, multidrug resistance protein) and drug-detoxifying enzymes, alterations in the membrane receptor, drug compartmentalization, dysfunction of the apoptotic signaling pathways, increase of DNA repair ability and alteration of cell cycle control (Gottesman, 2002).

The ABC transporter P-glycoprotein (P-gp) is localized in the plasma membrane of some cancer cells. This transporter is a target deeply implicated in the acquisition of drug resistance to a distinct variety of anticancer drugs (e.g. doxorubicin, etoposide, paclitaxel) by its ability to pump diverse drugs out of the cell. The promotor of the *MDR1* gene (that encode for P-gp) is upregulated by diverse cellular events, such as, DNA damage; serum starvation, amongst others. Most of these events are triggered by the cancer cell's initial response to anticancer drug toxicity (Tsuruo, Naito, et al., 2003). The link between drug cytotoxicity and P-gp activity validate this path as a key component for therapy targeting, nonetheless, it must be considered that this protein is also found in normal cells, as it is essential in the maintenance of homeostasis (Cordon-Cardo, O'Brien et al., 1990). Drug combination that allows interaction with multiple mechanisms can mitigate the deleterious effects of drug resistance.

1.5 Drug screening

Several approaches are employed in the search of new products with anticancer effect. Conventional screening includes bioactivity-guided screening and gene-guided screening. The National Cancer Institute (NCI) implemented a panel of sixty cancer cell lines with the aim to test compounds and select for further studying those that demonstrated promising bioactivity (Shoemaker, Scudiero et al., 2002). This method is a validation of a world reference use of bioactivity-guided screening (Covell, Huang, et al., 2007). Gene-guided screening where new genes responsible for the production of metabolites are searched based on their similarity with genes known to produce bioactive. This late methodology allows the identification of certain metabolites of interest from a vast number of organisms. Khan et al., (2010) from an initial number of 523 strains of marine bacteria was proceeded an analysis to evaluate the presence of 3-hydroxyl-3-methylglutaryl coenzyme A reduc-

tase (*hmgr*) gene, culminating in the isolation of two compound from two strain which demonstrated a cytotxic effect against HL-60 cell line (Khan, Izumikawa et al., 2010).

Once the large majority of known microorganisms are uncultured, resourcing to metagenomics seems a feasible method for a culture independent screening. By extracting the total DNA from an environment and introducing a gene in to a culturable host it is possible to access metabolites from organism not to be grown *in vitro*, and thus greatly increasing the ability to explore nature's capable portfolio in its wholeness. Terragine and acyltyrosines, a *Mycobacterium*-inhibiting antibiotic are such cases of the use of metagenomics in the discovery of new compounds (Brady, Chao, et al., 2002; Wang, Graziani, et al., 2000). The uncertainty regarding the correct expression of a gene cluster in the host is one of the difficulties facing the use of metagenomics for the screening of natural products (Xiong, Wang, et al., 2013).

Genomic strategies (Udwary, Zeigler, et al., 2007), combinatorial biology (McGlinchey, Nett, et al., 2008) and synthetic biology (Donia, Hathaway, et al., 2006) are also tools promptly utilized in the search of natural-derived products.

1.6 Emerging drugs from nature

Some diseases are currently facing changes in terms of treatment approach. In many cases, standard procedures are now presenting unsatisfactory results in comparison to their initial performance when were first implemented (Ciardiello and Tortora, 2008; Moore and Chaisson, 1999). For this reason, an immediate demand is rising for the search of new bioactive compounds and extracts. These novel bioactive molecules could ultimately translate into therapeutic drugs, and filling essential needs that current therapies are overpassing.

Nature has always been a source of novel and potent bioactive products with therapeutic applications. From 2001 to 2005, 23 nature-derived drugs reached the pharmaceutical market. The pharmaceutical industry has strongly invested in the bio-prospection within organisms, with an outcome of more than 60% of the approved drugs for cancer treatment from 1982 to 2002 being nature-derived (Newman, Cragg, et al., 2003). In 2007, 30 anticancer microorganism-derived drugs were at some stage of clinical trials. Such examples of nature's contribution are doxorubicin, daunomicin, bleomycin, mytomicin C, vincristine

and vinblastine which all prevail in the current available treatments for cancer (Jimeno, Faircloth, et al., 2004).

Notwithstanding, in the 1990s several limitations of natural products led to a disinvestment of the industry, which established as a priority the use of synthetic products. Amongst the negative aspects of natural product bio-prospection was its incompatibility with high throughput screening, which aimed to potentiate the bioactivity outcome in short periods of time, a situation not faced with synthetic products. Labor effort to obtain quality and uniform batches, quantitative scarcity for drug screening and preclinical tests, mimicking environmental conditions in order to stimulate the production of secondary metabolites *in vitro* similar to those in the wild and difficulty to manipulate large sized and complex chemical structure are some of the limitations faced (Glaser and Mayer, 2009). The stated reasons were concomitant with the decreased interest in exploring natural products for pharmaceutical purpose.

Moreover, secondary metabolites are as if inherently bioactive, as they have roles on defense over predation, as competitive advantage over other organisms or to communicate among a population. It is certain that a basic premise for these products is the ability to reach at a cellular level, much like drugs must do (Beutler, 2001). Nowadays with the technological impulse the search in nature for bioactive compounds is thriving and in expansion (Lam, 2007).

1.6.1. Marine sourced products

The marine environment occupies about 70% of the earth's surface and harvests an estimation of 95% of the world biodiversity (Ellis, 2001). Marine species have been evolving for about 3.5 million years alongside their chemical constituents that have provided these organisms the ability to surpass changes in salinity and pressure, temperature extremes, predation defenses and competition for space. It is possible to find even in the "simplest life forms" of the ocean the precursors of some of the immune components present in mammals (Prendergast, Lutty, et al., 1983; Scofield, Schlumpberger, et al., 1982). Marine-derived products are also a part of folk medicine in different cultures, as for example, the use of seaweed-based remedies for the treatment of pain, cancer and other illnesses (Ruggieri, 1976). In the last decades the interest and the accessibility towards marine products has greatly increased partially due to the dramatic improvement of diving techniques. Since then, a myriad of marine natural products with advantages and novel

mechanisms of action regarding currently used products have been discovered (Mayer and Gustafson, 2003) (Table 2). Manoalide, an unusual nonsteroidal sesterterpenoid, demonstrated to be first inhibitor of phospholipase A with potent analgesic and anti-inflammatory effect, aspiring to be used in multiple disorders (Soriente, Rosa, et al., 1999). Ecteinascidin-743, or trabectedin (ET-743, trademark Yondelis®) is a tetrahydroisoquinoline alkaloid and represents a successful symbol of a marine-derived drug. Research, development and marketing were responsibility of the pharmaceutical company PharmaMar Inc. (Madrid, Spain). ET-743 is derived from a tunicate, *Ecteinascidia turbinate*, present in the Caribbean and Mediterranean Sea (Rinehart, Holt, et al., 1990). Its mechanism of action, although not fully decoded, it is known to interact with the DNA, inhibiting transcription, and also inactivating drug resistance mechanisms (Kanzaki, Takebayashi, et al., 2002; Minuzzo, Mantovani, et al., 1999). ET-743 is approved in Europe for the treatment of soft tissue sarcoma and ovarian cancer and is undergoing different phases of clinical trials for breast, lung, prostate and pediatric cancer (Verweij, 2009; Yap, Carden, et al., 2009). Some of the biosafety advantages for patients are the absence of mucositis, alopecia, neurotoxicity, cardiotoxicity or cumulative toxicities; these represent some of the most common side effects of current chemotherapeutic agents (Mayer, Glaser, et al., 2010).

Table 2 – Marine derived anti-cancer drugs in the year of 2010. Adapted from (Mayer, Glaser, et al., 2010).

Clinical status	Compound	Trademark	Origin organism	Chemical family
Marketed	Cytarabine, Ara-C	Cytosar-U®	Sponge	Nucleoside
	Trabectedin (ET-743)	Yondelis®	Tunicate	Alkaloid
Phase III	Eribulin Mesylate (E7389)	n.d	Sponge	Macrolide
	Soblidotin(TZT 1027)	n.d	Bacterium	Peptide
Phase II	PM1004	n.d	Bacterium	Peptide
	Plitidepsin	Aplidin®	Tunicate	Depsipeptide
	Plinabulin(NPI-2358)	n.d	Fungus	Diketopiperazine
	DMXBA (GTS-21)	n.d	Worm	Alkaloid
	Tasidotin,Synthadotin (ILX-651)	n.d	Bacterium	Peptide
	Elisidepsin	Irvalec®	Mollusc	Depsipeptide
Phase I	Bryostatin 1	n.d	Bryozoa	Polyketide
	Hemiasterlin	n.d	Sponge	Tripeptide
	Marizomib (Salinosporamide A; NPI-0052)	n.d	Bacterium	Beta-lactone-gama lactam

n.d – not defined

1.6.2. Marine invertebrates as sources of bioactive compounds

Sponges (Phylum Porifera) are marine invertebrates that are widely distributed world-wide. They inhabit from the intertidal zone to deeper regions and are present not only in tropical waters, but also in temperate and freshwaters (Bergquist, 2001). Sponges are divided into 3 classes (Calcarea, Demospongiae and Hexactinellida), being the Demospongiae the most representative class. Regarding the simple anatomical structure of this sessile organism, an array of metabolites is employed by sponges to proclaim their space when competing with other organisms willing to occupy their surroundings (Cheng,

Rifkin, et al., 1968). In spite of sponges possessing specialized structures that interplay a role in their self-defense, chemical intervenients named secondary metabolites are a complementary system of major importance for the competitive oceanic ground battle (Wang, 2006). The pioneering studies regarding sponge secondary metabolites were undertaken by Bergmann and Feeney in 1951, and lead to the isolation of nucleosides from *Tectitethya crypta* (once *Cryptotethya crypta*), which were later confirmed to possess antiviral properties (Bergmann and Feeney, 1951). Purification of sponge secondary metabolites allowed the identification of macrolides, porphyrins, terpenoides, aliphatic cyclic peroxides, sterols, and amino acids. Arabinosyl cytosine (Ara-C), a current anticancer drug, Manolide (from *Luffariella variabilis*) an anti-inflammatory and 9-β-D-arabinofuranosyladenine (Ara-A) an anti-viral, are brief examples of drugs already available in the market (Thakur and Muller, 2004).

Marine invertebrates, such as sponges, live in close relation with a microbiota community. This symbiosis occurs with bacteria, fungi, microalgae, archae and virus and the symbiont can occupy up to 40% of the total sponge volume (Wilkinson, 1978). The symbiont can furnish the host with some required nutrients (e.g. carbon fixation by cyanobacteria). Additionally, the symbiont may also contribute to the increase of the host's defense mechanisms through the production of secondary metabolites (Webster and Taylor, 2012).

1.6.3. Marine fungi

Marine microorganisms represent about 90% of the ocean's biomass (Boopathy, Kathiresan, et al., 2010). Terrestrial fungi have been intensively explored and have presented true proof in terms of biotechnological applicability. However, marine fungi have gained notoriety and are also beginning to be more thoroughly explored, consequently, promising results are starting to appear. Some of the secondary metabolites extracted from marine fungi have been shown to possess anti-oxidant, anti-proliferative, anti-inflammatory, anti-metastasis and pro-apoptotic activity (Kim and Dewapriya, 2013).

From the halotolerant marine fungus strain *Aspergillus variecolor B-17* two new quinone compounds were isolated, variecolorquinones A and B, presenting selective cytotoxicity against a series of cancer cell lines. Variecolorquinone A inhibits the proliferation of A549 cells (IC_{50} of 3.0 μM), and variecolorquinone B inhibits the proliferation of HL-60 and P388 cells (IC_{50} of 1.3 μM and 3.7 μM, respectively) (Wang, Zhu, et al., 2007).

Dicitrinone B was first isolated from the marine-derived fungi *Penicillium citrinum* by Du et al., 2010, demonstrating cytotoxicity against HL-60, A549, BEL-7402 and MOLT-4 cells with IC_{50} values of 6.5 μM, 53.9 μM, 39.8 μM and 6.0 μM, respectively. This compound inhibits the proliferation of HL-60 cells by arresting cell cycle at the G2/M phase (Du, Zhang et al., 2010). Recently, this compound was tested in A375 and was demonstrated to induce apoptotic pathways by activation of caspase 3, 8 and 9 (Chen, Gong et al., 2014).

The secondary metabolite (−)-phenylahistin isolated from the marine and terrestrial fungus *Aspergillus ustus* showed anti-proliferative effects by Kanoh *et al.* in 1997 acting by inhibition of tubulin polymerization inhibiting cell cycle in G2/M phase. After a series of chemical modifications by Yoshio et al., in 2013 in order to achieve a water-soluble pro-drug, this compound was tested in a phase II clinical trial by Nereus Pharmaceutical (San Diego, CA, USA) (Hayashi, Yamazaki-Nakamura, et al., 2013; Kanoh, Kohno, et al., 1997; Newman and Cragg, 2014).

1.6.3.1. *Neosartorya* genus

Neosartorya species are closely related with the *Aspergillus* genus (Rydholm, Szakacs, et al., 2006). Compounds isolated from the *Aspergillus* genus have demonstrated anti-cancer activity both *in vitro* and *in vivo*, as evaluated by Bladt et al. 2013 (Bladt, Frisvad, et al., 2013). While the bioactivity of the secondary metabolites produced by *Neosartorya* species remains less explored, a greater potential may have been overlooked. Recently, several research groups have been dedicated to understanding which biotechnological applications can be developed from these metabolites. Present hereon are some of the works that demonstrated anticancer effects of secondary metabolites isolated from *Neosartorya* species.

Eamvijarn et al., (2013) isolated several compounds (aszonalenin, acetylaszonalenin, aszonapyrone B, 13-oxofumitremorgin B and aszonapyrone A) from the soil fungus *Neosartorya fischeri* (KUFC 6344), aszonapyrone A from the diseased coral-derived fungus *Neosartorya laciniosa* (KUFC 7896) and an analogue from chevalone C from the marine sponge-associated fungus *Neosartorya tsunodae* (KUFC 9213). The cytotoxicity of the compounds was tested in a panel of three cancer cell lines (MCF-7, NCI-H460 and A375-C5) with GI_{50} values ranging from 10.2 μM to >150 μM (Eamvijarn, Gomes, et al., 2013). In another study, Eamvijarn et al., 2012 isolated seven compounds (two of which reported

as new compounds) from the fungus *Neosartorya pseudofischeri.* Two of the already known compounds demonstrated an anti-cancer effect. One compound exhibited growth inhibitory activity, comparable to the effect exerted by etoposide and carboplatin in a panel of cancer cell lines. Another compound displayed cytostatic effect in U373 and A549 cells (Eamvijarn, Kijjoa, et al., 2012).

Buttachon et al., 2012 isolated fourteen new compounds from the soil fungus *Neosartorya siamensis* (KUFC 6349). Eight of these compounds were evaluated in regard to growth inhibitory activity in U373, Hs683, A549, MCF-7 and SK-MEL-28 cancer cells, having shown IC_{50} values ranging from 39 μM to >100 μM (Buttachon, Chandrapatya, et al., 2012).

Tan et al., (2011) obtained two new compounds (fischeacid and fischexanthone) and eight already known, from the marine-derived fungus *Neosartorya fischeri* strain $1008F_1$. The anti-cancer bioactivity of the compounds at a concentration of 200 μg/ml was assessed in SGC-7901 and BEL-7404 cancer cell lines, showing cell proliferation inhibition ranging from 11.3% to 89.8%. Antiviral (tobacco mosaic virus) effect was also reported from some compounds found in this fungus (Tan, Ouyang, et al., 2011).

Kijjoa et al. (2011) isolated 3 compounds (sartoryglabins A-C) from the fungus *Neosartorya glabra*, which demonstrated strong to moderate effect against MCF-7 cells, and weak or no effect against NCI-H460 and A375-C5 cells (Kijjoa, Santos, et al., 2011).

From past to present cancer has been faced as a challenge to the scientific and medical community. Great advances have been made in the biology of cancer allowing the improvement of early detection of cancer and the treatments available. However the challenge remains and the multiple thematic approaches need to be further developed. Drug screening of new compounds derived from marine organisms could provide a significant improvement to current therapeutics, as demonstrated by several recent novel products.

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CHAPTER 2

EXTRACTS FROM *Neosartorya* (FUNGI) SPECIES
EXHIBIT ANTI-PROLIFERATIVE ACTIVITY
WITH CELL DEATH INDUCTION IN
COLON, BREAST AND SKIN CANCER CELL LINES

This chapter comprises the following original manuscript submitted to *Phytotherapy Research:*

Ramos A.A.*, **Castro-Carvalho B.***, Prata-Sena M., Dethoup T., Buttachon S., Kijjoa A. & Rocha E. (2014) Extracts from *Neosartorya* (fungi) species exhibit anti-proliferative activity with cell death induction in colon, breast and skin cancer cell lines.

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2.1. Manuscript

Abstract

Marine and soil fungi are promising sources for bioprospection of novel compounds with applications regarding human health. This study aimed to assess the *in vitro* anticancer activities of crude ethyl acetate extracts of marine-derived fungi *Neosartorya tsunodae* KUFC 9213 (E1) and *Neosartorya laciniosa* KUFC 7896 (E2) and soil fungus *Neosartorya fischeri* KUFC 6344 (E3), on a panel of seven human cancer cell lines. Initially, MTT assay was performed after 48 h treatments with diverse concentrations of extracts to determine the IC_{50} for each extract per cell line. Effects on DNA damage, clonogenic potential and ability to induce cell death were also assessed. Extract E1 did not exhibit anti-proliferative effects. Extract E2 decreased the clonogenic potential in HCT116, A375, MCF7 and HT29 cells, whereas extract E3 showed such effect in HCT116 and MCF7 cells. Both extracts increased DNA damage in some cell lines. Extract E2 induced cell death in HT29, HCT116, MCF7 and A375 cells, while extract E3 increased death in MCF7 and HCT116 cells. Results reveal that extracts E2 and E3 have anticancer activities in human colon carcinoma, breast adenocarcinoma and melanoma cells, validating the interest for future identification of the compounds and molecular targets involved in the anticancer activity.

Keywords: Marine-derived fungi; anticancer activity; anti-proliferative activity; cancer cell lines; cell death; *Neosartorya* species.

1. Introduction

Cancer is one of the main causes of death in developed countries and is gaining lead in developing ones. Its incidence is steadily increasing in parallel with population growth and aging, as well as with some environmental and lifestyle factors (Jemal et al., 2011). The individual's gene pool influence many be high and must be also considered (Frank et al., 2014). Since cancer has high human/social costs, all sorts of strategies to prevent or treat cancer are desirable. Lifestyle changes, early detection and new treatments are key to prevent and reduce the cancer incidence (Jemal al., 2011). Considering the (*i*) increasing frequency of neoplastic lesions, (*ii*) the resistance acquired to conventional common-use therapeutic drugs (Holohan et al., 2013), (*iii*) cross resistance to structurally dissimilar and unused anticancer drugs (Iseri et al., 2009), and (*iv*) undesirable side effects of current chemotherapeutic drugs, new bioactive substances are needed.

The marine environment is a prosperous and still underexploited resource of bioactivnatural compounds (Mayer et al., 2010). Bioprospection of the seas has brought to light numerous novel compounds that have been pinpointed as potential therapeutical agents, especially by demonstrating bioactive profiles with anticancer, anti-inflammatory, anti-viral, anti-angiogenic, antioxidant and anti-adhesion activity (García et al., 2013; Lind et al., 2013; Mayer et al., 2013, Von and Vollmar, 2013). Trabectedin (Yondelis®, ET-743), is one of the few marine-derived drugs approved for clinical use as an anticancer medicine, more remain under clinical trial phases, as is the case of brentuximab vedotin (Adcetris®) and PM00104 (Zalypsis®) (Newman and Cragg, 2014) and of plitidepsin (Aplidin®) (Ribrag et al., 2013).

Marine-derived fungi may be of key relevance in the quest for biopharmaceuticals since fungi produce metabolites with interesting bioactive activities (Greve et al., 2010). Moreover, marine fungi may overcome the frequent limitation of production shortfall, since they have the potential to be easily produced under laboratorial/industrial conditions, and are therefore good candidates for compound extraction under mass-production. Several compounds from marine fungi presented cytotoxic and cytostatic effects on human cancer cell lines (Pejin et al., 2013). Moreover, in extended studies compounds isolated from marine fungi have been found to target different pathways involved in proliferation and cell death mechanisms and other hallmarks of cancer. Such an example is cryptosphaerolide, from the marine-derived ascomycete fungal strain CNL-523 of the genus *Cryptosphaeria* sp., which induced apoptosis by the inhibition of Mcl-1 protein, member of the essential cell death regulatory proteins Bcl-2 family

(Jasperson et al., 2010). The anthraquinone SZ-685C, from *Halorosellinia* sp., a mangrove endophytic fungus, had large cytotoxic effects in 6 cancer cell lines, inducing apoptosis by inhibition of Akt/FOXO pathway (Xie et al., 2010). Peribysin D has been identified as being an exceptional cell-adhesion inhibitor in HL-60 cells to HUVEC (human umbilical endothelial cell) and may be a key in preventing metastasis (Yamada et al., 2004).

More recently, fungus species of the *Neosartorya* genus, which represent sexual forms of the *Aspergillus* species from the *Fumigati* section, have gained interest as potential sources for the isolation of potential anticancer compounds (Tan et al., 2011; Eamvijarn et al., 2013; Buttachon et al., 2012). With this rationale in mind, the aim of this study was to assess the *in vitro* anticancer activity of the crude extract of three fungi species, marine-derived fungi *Neosartorya tsunodae* KUFC 9213 (**E1**) and *Neosartorya laciniosa* KUFC 7896 (**E2**) and soil fungus *Neosartorya fischeri* KUFC 6344 (**E3**) on a panel of seven human cancer cell lines: colon carcinoma (HT29 and HCT116), liver hepatocellular carcinoma (HepG2), breast adenocarcinoma (MCF7), malignant melanoma (A375), lung carcinoma (A549) and glioblastoma (U-251) cells.

2. Material and Methods

2.1. Reagents

Doxorubicin (Dox), Dulbecco's Modified Eagle Medium (DMEM), Minimum Essential Medium Eagle medium (MEM), Roswell Park Memorial Institute medium (RPMI), McCoy's 5A Modified medium, penicillin/streptomycin, trypsin solution, 4,6-diamidino-2-phenylindole (DAPI) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dimethylsulfoxide (DMSO) was purchased from AMRESCO LLC (Solon, SO, USA). Fetal bovine serum (FBS) was purchased from Biochrom KG (Berlin, Germany). All other reagents and chemicals used were of analytical grade.

2.2. Fungal Material

Neosartorya tsunodae (KUFC 9213) was isolated from the marine sponge *Aka coralliphaga*, which was collected from the coral reef of the Similan islands, Phagna province, Thailand, by scuba diving at 10 m depth, in April 2010 and the sponge was identified as described by Eamvijarn et al. (2013). The pure cultures were deposited as

KUFC 9213 at Kasetsart University Fungal Collection, Plant Pathology Department, Agriculture Faculty, Kasetsart University, Bangkok, Thailand.

Neosartorya fischeri (KUFC 6344) was isolated from coastal forest soil at Samaersarn island Chonburi Province, Thailand, in November 2008 and identified as described by Eamvijarn et al. (2013). The pure cultures were deposited as KUFC 6344 at the Mycology Laboratory, Plant Pathology Department, Agriculture Faculty, Kasetsart University, Bangkok, Thailand, and as IFM 59696 at the Medical Mycology Research Center, Chiba University, Japan.

Neosartorya laciniosa (KUFC 7896) was isolated from a diseased coral (ulcerative white spot in *P. lutea*) at Ao Nuan Lan island, Chonburi Province, in the Thailand Gulf, in May 2010 and identified as described by Eamvijarn et al. (2013). The pure cultures were deposited as KUFC 7896 at Kasetsart University Fungal Collection, Plant Pathology Department, Agriculture Faculty, Kasetsart University, Bangkok, Thailand, and as MMERU 01 at Microbes Marine Environment Research Unit, Environmental Science Division, Science Faculty, Ramkhamhaeng University, Bangkok, Thailand.

2.3. Crude ethyl acetate extracts preparation

Crude ethyl acetate extracts were made as described by Eamvijarn et al. (2013). Briefly, marine fungi were cultured in five 90 mm Petri dishes with malt extract agar (MEA) (for the strains KUFC 9213 and KUFC 7896) or with potato dextrose agar (for the strain KUFC 6344) for one week. Then Erlenmeyer flasks containing rice and water were autoclaved, inoculated with the respective fungus and incubated for 30 days at 28º C. The mouldy rice was macerated in ethyl acetate, filtrated, and then the two layers were separated with a separatory funnel, and the ethyl acetate solution was concentrated at a reduced pressure.

2.4. Cell lines

HT29, HCT116 cells were kindly provided by Prof. Carmen Jerónimo, from CI-IPO, Porto. HepG2 cells were kindly provided by Prof. Rosário Martins, from ESTSP and CIIMAR, Porto. A375, A549, U-251 and MCF7 cell lines were obtained from European Collection of Cell Cultures (ECACC). Cells were maintained as monolayer cultures in DMEM (HT29, A375 and A549 cell lines) in MEM (HepG2, U-251 and MCF7 cell lines) or RPMI for HCT116 cell line. The mediums were supplemented with 10% FBS, 1% antibiotic solution (100 U/ml penicillin and 100 µg/ml streptomycin), 10 mM HEPES and 0.1 mM sodium pyruvate. Cells were maintained in an incubator with a humidified at-

mosphere of 5% CO_2 at 37º C. Cells were trypsinised when approaching confluence and medium was changed every two days. For experiments, the test extracts and Dox were dissolved in DMSO (final concentration < 0.5%) and controls received DMSO only.

2.5. Evaluation of cell viability/proliferation by MTT Assay

Effects in cell viability/proliferation were evaluated by the MTT reduction assay (Ramos et al., 2008). Briefly, cells were seeded at a density of $0.8x10^4$ to $1x10^4$ cell/well in 96-well plates and incubated for 24 h in 5% CO_2 at 37º C. After adhesion, cells were exposed to fresh medium containing varying concentrations of fungi extracts (0.1, 1, 10, 100 and 200 µg/ml) or Dox (0.001, 0.01, 0.1, 1 and 10 µM) that was used as positive control. After 48 h of treatment, MTT solution at final concentration of 0.5 mg/ml was added and incubated for 2 h at 37º C. Ethanol-DMSO (1:1) (v/v) solution was used to dissolve the formazan crystals and absorbance (A) was measured at 570 nm in a microplate reader (Multiskan EX, Labsystems, USA). The number of viable cells in each well was estimated by the cell's capacity to reduce MTT and produce formazan crystals (Ramos et al., 2008). The concentration of extract or Dox that inhibits cell viability by 50% (IC_{50}) was calculated by analysing dose-response data with GraphPad Prism v5.0 software (GraphPad Software, La Jolla, CA, USA).

To evaluate effects on cell proliferation, absorbance at the beginning of incubation (*t* = 0 h) was subtracted from all the experimental conditions, including the negative control (cells without any test extract) at the end of treatment (t = 48 h). Cell proliferation and cell inhibition percentages were calculated according to the following equations, where A is absorvance; iC is initial control and fC is final control.:

$$\text{Cell proliferation (\%)} = [(A_{sample} - A_{iC})/(A_{fC} - A_{iC})] \text{ X } 100$$

$$\text{Cell inhibition (\%)} = 100 - \text{Cell proliferation}$$

In this way, whereas negative values for cell proliferation imply direct cytotoxic effects of extracts, positive values (between 0 and 100%) imply inhibition of cell proliferation. The results correspond to the mean of at least six independent experiments; each carried out in duplicate. For the following assays, only fungi extracts inducing an IC_{50} inferior to 200 µg/ml and without cytotoxic effects were used.

2.6. Evaluation of cytostatic effect by clonogenic cell survival assay

Long-term cytostatic effect was evaluated by the clonogenic cell survival assay. Cells were seeded in 24-well plates with a density of 1 x 10^6 cells/ml and after adhesion cells were incubated with extracts and Dox at IC_{50} concentrations (determined above). After 48 h of treatment cells were harvested by trypsinization and then the survival cells seeded in 12-well plates at a density of 200 cells per well. Cells were incubated with drug-free medium for 10 days under normal culture conditions. After the incubation period, cells were washed with PBS and fixed in situ with 4% paraformaldehyde (in PBS) for 15 min at room temperature. Then, cells were stained with 0.05% crystal violet for 30 min at room temperature washed with distilled water and the plates were left to dry. In each treatment condition, colonies made of more than ~ 50 cells were quantified by a stereomicroscope (Leica, ZOOM 2000) and the plating efficiency (PE) and surviving fraction (SF) were calculated according to the following equations (Franken et al., 2006; Munshi et al., 2005): PE = number of colonies counted/number of seeded cells and SF = (PE of treated cells/PE of control) x 100.

2.7. Evaluation of genotoxic effect by single cell electrophoresis assay (Comet Assay)

Effects on DNA damage were evaluated by the single cell electrophoresis assay or comet assay (Collins et al., 2008; Ramos et al., 2010). Briefly, cells were seeded at a density of 0.1 x 10^6 cells/ml in 24-well plates. After adhesion, cells were treated with the respective IC_{50} concentrations of fungal extracts and Dox for 4, 24 and 48 h at 37º C. By the end of each treatment, 2 x 10^4 cells were collected per sample, mixed with a 0.5% (w/v) low melting agarose and mounted on slides previously coated with a 1% (w/v) normal melting agarose. Up to this point, all samples were maintained at 4ºC to minimize further DNA repair. Slides were placed in lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, pH 10) plus 1% (v/v) Triton X-100 for 1h at 4º C and then incubated in an alkaline electrophoresis buffer (300 mM NaOH, 1 mM Na_2EDTA, pH 13) for 40 min at 4ºC to allow the DNA to unwind. After a 20 min electrophoresis at 21 V, slides were washed, fixed with 100% ethanol, and dried at room temperature. Afterwards slides were stained with DAPI solution at 1µg/ml, observed under a fluorescence microscope (Olympus IX71) and the CometScore® software (CometScore, TriTek Corp.) was used to calculate the parameter of percentage of tail intensity.

2.8. Evaluation of cell death induction by nuclear condensation assay

Effects of test extracts on the induction of cell death were evaluated by the nuclear condensation assay. Cells were seeded at a density of 1 x 10^6 cells/ml on 24-well plates and incubated for 24 h at 37ºC. After adhesion, cells were treated with the respective IC_{50} concentrations of fungi extracts and Dox and left to incubate for 48 h. In each well, adherent and detached cells were collected, washed, centrifuged and fixed with 4% (w/v) paraformaldehyde in PBS for 15 min at 37ºC. Samples were stored at 4ºC until further use. On the day of experiment, cells were mounted on polylysine-treated slides using a Cell Spin Cytospin centrifuge and left to air dry. Ensuing, the slides were thrice-washed with PBS for 5 min each and incubated for 10 min in the dark with DAPI (1µg/ml) for nuclei staining. The cells with condensed nuclei were observed using a fluorescence microscope (Olympus IX71) and at least 300 cells were scored per sample. The percentage of cells with condensed nuclei was determined according to the following equation: % of cells with condensed nuclei = (Number of cells with nuclear condensation/Total number of cells) x 100.

2.9. Statistical analysis

Results are expressed as mean ± SD from at least 3 independent experiments. Statistical tests were performed by one-way ANOVA, followed by the post-hoc Newman-Keuls multiple comparison test or Dunnett's test or by the Student's *t*-test, using the software GraphPad Prism v5.0 (GraphPad Software, La Jolla, CA, USA). P values ≤ 0.05 were considered statistically significant.

3. Results and Discussion

As a first step to ground further research, the extracts were screened for anti-proliferative activity against HepG2, HT29, HCT116, A375, A549, MCF7and U251 cancer cell lines. After 48 h of cell exposure to a range of concentrations of the extracts of marine fungi and Dox (positive control), effects on cell viability/proliferation were measured by MTT reduction assay. Impacts of extract and Dox on cell viability were summarized in Table 1, by IC_{50} values that correspond to the concentration that was able to cause a 50% inhibition of viability. At the tested concentrations, the *Neosartorya tsunodae* extract (**E1**) did not decrease cell viability in any of the tested cell lines (in all cases the IC_{50} was higher than 200 µg/ml). Despite, Eamvijarn et al. (2013), which in a recent work demonstrated that sartorypyrone B, isolated from the ethyl acetate extract of *Neosartorya tsunodae* showed an anti-proliferative effect on MCF7, A375-C5 and

NCI-H460 cells; however in our work crude ethyl acetate extract **E1** did not demonstrate anti-proliferative effects.

The *Neosartorya laciniosa* extract (**E2**) was found to inhibit four of the human cancer cell lines tested, namely in HT29, HCT116, A375 and MCF7 cells. The IC_{50} value for that extract was 139, 141, 179 and 200 µg/ml in HCT116, A375, MCF7 and HT29, respectively, showing that HCT116 and A375 cells were the most sensitive to **E2**.

Neosartorya fischeri extract (**E3**) also presented relevant inhibitory activities ($IC_{50} \leq$ 200 µg/ml) on HCT116 and HT29 (IC_{50} of 189 µg/ml and 196 µg/ml, respectively) and MCF7 (IC_{50} of 189 µg/ml), although less active than extract **E2**. Doxorubicin was used as a positive control, and was shown to decrease cell viability in all tested cell lines in a dose-dependent manner; the IC_{50} values range from 0.11 µM to 1.55 µM. The U251, HT29 and A549 cell lines were the most resistant ones, with an IC_{50} of 1.55, 0.87 and 0.54 µM, respectively.

Table 1 – IC_{50} values of extracts from *Neosartorya tsunodae* (E1), and *Neosartorya laciniosa* (E2) *Neosartorya fischeri* (E3) in seven cell lines determined by MTT assay.

	IC_{50} (95% confidence interval)			
Cell lines	E1 (µg/ml)	E2 (µg/ml)	E3 (µg/ml)	Doxorubicin (µM)
HepG2	>200	>200	>200	0.11 (0.07-0.17)
HT29	>200	**200** (177-226)	**196** (167-229)	0.87 (0.54-1.39)
HCT116	>200	**139** (120-164)	**189** (166-214)	0.13 (0.09-019)
U251	>200	>200	>200	1.55 (0.70-2.50)
A549	>200	>200	>200	0.54 (0.30-0.94)
A375	>200	**141** (120-166)	>200	0.12 (0.09-0.16)
MCF7	>200	**179** (154-205)	**189** (156-225)	0.37 (0.27-0.50)

IC_{50} values are the mean at least 6 independent experiment each in duplicate. Doxorubicin was used as a positive control.

As proposed, the effects on cell proliferation were also inferred from the MTT results. As shown in Figure 1A, extract E2 significantly inhibited cell proliferation in HT29, HCT116, A375 and MCF7 in a dose-dependent manner. Also extract **E3** decreased cell

proliferation in a dose-dependent manner in HT29, HCT116 and MCF7 cells (Figure 1B). For concentrations higher than 200 µg/ml, both extracts induced a cytotoxic effect (Figure 1). The percentage of inhibition of cell proliferation of extracts **E2** and **E3** at the same concentration (e.g., 100 µg/ml) are summarized in Table 2. In HCT116 and MCF7 cells, extract E2 inhibited cell proliferation by 45% and 69%, while extract E3 inhibited proliferation by 26% and 39%, respectively. Contrarily, HT29 cells showed the lowest rates of inhibition of cell proliferation for both extracts (**E2** - 24% and **E3** - 19%).

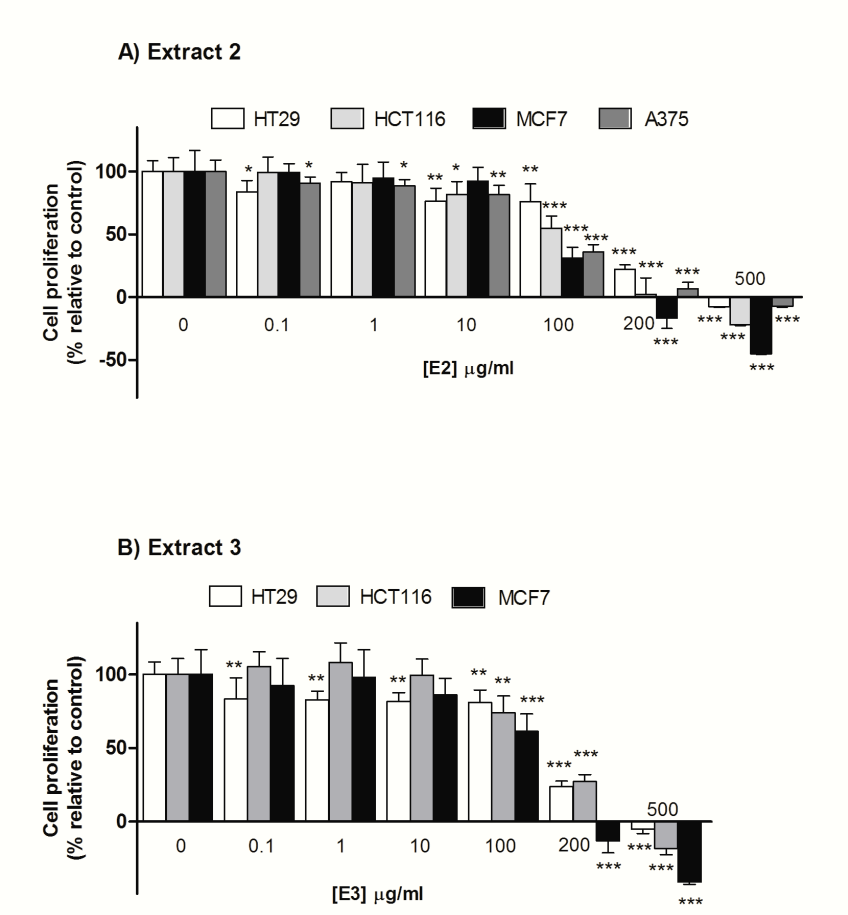

Figure 1. Dose-response effects of extracts E2 (A), E3 (B) on cell proliferation in HT29, HCT116, MCF7 and A375 cells after 48 h, evaluated by MTT assay. Results are expressed as mean + SD of at least six independent experiments, in duplicate. Significant differences (* $P \leq 0.05$; ** $P \leq 0.01$ and *** $P \leq 0.001$), were determined by the post-hoc Newman-Keuls multiple comparison test.

Table 2 – Effect of 100 µg/ml E2 and E3 extracts on cell proliferation (percentage of cell inhibition) after 48h of incubation.

Cancer cell lines	Percentage of cell inhibition (CI)	
	E2 extract	E3 extract
HT29	24	19
HCT116	45	26
MCF7	69	39
A375	64	n.d

n.d – not determined

Attending to changes in IC_{50} values and in percentages of cell inhibition between cell lines, it seems that the extracts may act by different pathways, depending of the genetic characteristics of each cell line. Particularly, this can be suggested based on the fact that the drug resistance demonstrated by several types of cancers, namely colorectal cancer, is often related with p53 mutations (Ravizza et al., 2004). In our work, both extracts (**E2** and **E3**) were more bioactive (showed lower IC_{50} values) in cell lines with wild-type p53, however, they also showed an anti-proliferative effect in the HT29 cell line (p53 mutant), which may indicate that the extracts may act by p53-dependent and independent mechanisms.

In the cell lines most sensitive to the effect of the extracts (HCT116, A375 and MCF7), extract **E2** was more bioactive than extract **E3**, which may reflect differences in the extracts' chemical composition. Recently, Eamvijarn et al. (2013) isolated compounds from the ethyl acetate extract of the fungi *N. fischeri* and *N. laciniosa* and demonstrated the anti-proliferative effect of 13-oxofumitremorgin B (2) and sartorypyrone A (3) isolated from *N. fischeri*, aszonapyrone A (4b) isolated from *N. fischeri* and *N. laciniosa* in MCF7, NCI-H460 and A375-C5 cancer cell lines. GI_{50} values ranged from 10.2 µM to 123.3 µM. Aszonapyrone A, present in both extract **E2** and **E3,** was considered the most potent compound with GI_{50} around 10 µM for all the cell lines tested. Tryptoqivaline L, that was also isolated from the fungus *N. laciniosa,* showed cytotoxic effects in MCF7 cells (Sodngam et al., 2014). According to our data, these compounds and others may be responsible for the anti-proliferative effect of the extract **E2** and **E3**.

Several marine compounds have shown strong anti-proliferative effects. Aspergiolide B, an aromatic polyketide isolated from marine-derived *Aspergillus glaucus*, had

a strong cytotoxic effect on A549 and HL60 cell lines, with respective IC_{50} values of 0.24 μM and 0.51 μM (Du, Zhu et al., 2008). The cyclic pentadepsipeptide zygosporamide, isolated form marine-derived *Zygosporium masonii*, revealed a potent cytotoxic effect in a panel of 60 cell lines, with a median growth inhibition (GI_{50}) of 9.1 μM, and presenting a specificity to RXF 393 and SF-268 cancer cell lines, with a GI_{50} of 5.0 and 6.5 nM, respectively (Oh et al., 2006).

Our extracts revealed anti-proliferative effects throughout the tested concentrations, demonstrating a dose-response effect. Such consequences are in line with morphological changes observed by phase contrast microscope after treatments either with extracts or Dox (data not shown). The main changes were decreased cell density, increasing of rounded and detached cells (that may indicate cell death), and cell shrinkage.

Only extracts with an $IC_{50} \leq 200$ μg/ml and that did not induce direct cytotoxic effect were took for further testing, namely to assess the clonogenic potential, the percentage of DNA damage, and the ability to induce cell death. Therefore, extracts **E2** and **E3** were selected for HT29, HCT116, MCF7 and A375 (only for **E2**) cell lines.

A clonogenic assay was performed in order to assess whether the fungi extracts possess a long-term cytostatic effect on the ability of a single cell to proliferate into a viable colony (Sumantran, 2011). Cells previously exposed to extracts for 48 h were allowed to grow in fresh medium for 10 days, dyed, and afterward all colonies formed by more than 50 cells were counted. As shown in Figure 2, extract E2 presented a significant decrease of clonogenic potential by over 90% in all tested cell lines, more specifically in HT29 (94%), HCT116 (97%), A375 (99%) and MCF7 (97%) cancer cell lines, compared with the (DMSO) control. Extract E3 also exhibited a strong reduction of the proliferative ability of a single cell to form a viable colony in MCF7 (93%) and a moderate inhibition in HCT116 (25%) cells. Doxorubicin showed an almost total inhibition of clonogenic potential in all cell lines. These results show that both extracts **E2** and **E3** (except extract E3 in HT29 cells) not only affect short term cell proliferation, in line with the MTT reduction assay, but also impact over indefinite proliferation mechanisms.

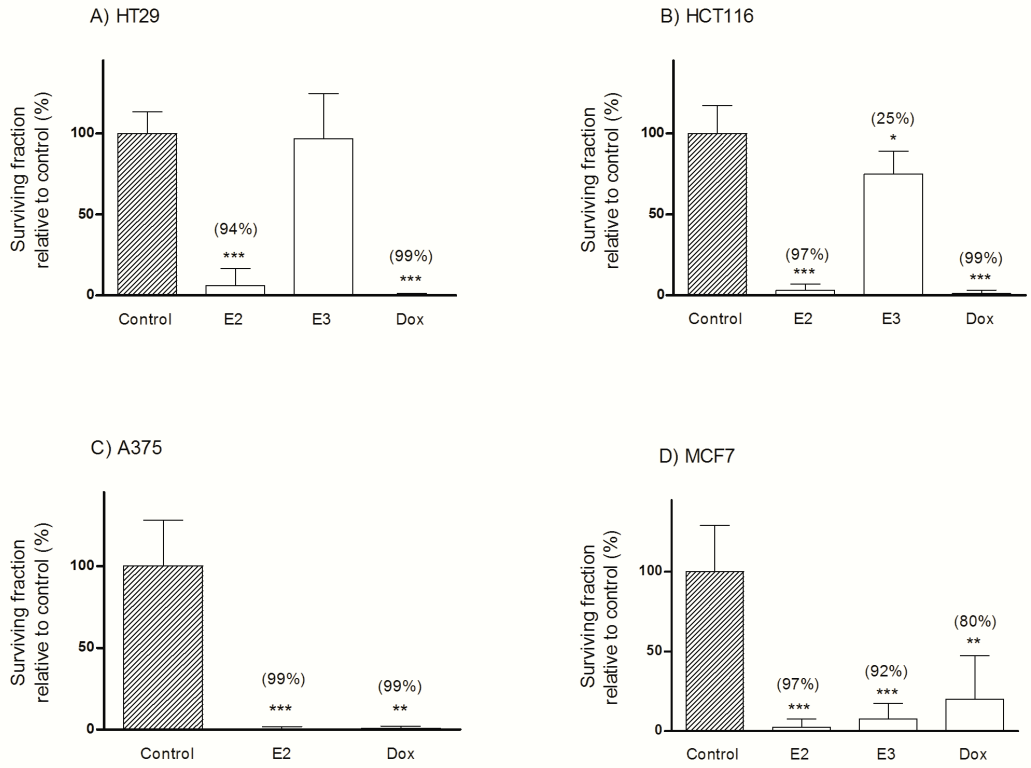

Figure 2. Effect of extract E2 and E3 on the clonogenic potential of human tumour cell lines; (A) HT29 cell line; (B) HCT116 cell line; (C) A375 cell line and (D) MCF7 cell line. Percentages in brackets are shown as the increase in relation to negative control. Results are expressed as mean + SD of at least three independent experiments. Significant differences (* $P \leq 0.05$; ** $P \leq 0.01$ and *** $P \leq 0.001$) when compared with negative control were determined by one-way ANOVA, followed by the post-hoc Dunnett's test.

DNA damage was determined quantitatively by a single-cell electrophoresis assay or comet assay (Figure 3). HCT116, MCF7 and A375 cells were exposed to IC_{50} concentrations of extracts **E2** and **E3** (Table 1) and Dox as a positive control for 4, 24 and 48 h prior to the comet assay. Most of the tested cancer cell lines presented a consistent increase in DNA strand breaks by one or more of the extracts, with exception to HT29 colon cell line (data not shown); in which neither extract (**E2** and **E3**) produced effect. In MCF7 cells, extracts **E2** (41.62 ± 8.18%) and **E3** (28.68 ± 2.18%) significantly induced DNA damage relative to untreated control cells (15.94 ± 3.22%). Moreover, in A375 cells, extract **E2** (27.17 ± 7.70%) also induced significant DNA damage in relation to the negative control (5.25 ± 1.05%). Extract **E2** induction of DNA damage was also clear in HCT116 cells, with a significant induction by 25.41 ± 5.40% over 14.43 ± 7.02% of the untreated cells, while extract E3 did not exhibit a significant difference as

to the negative control. Drugs that are able to induce consistent DNA damage may lead to cell death induction if the DNA damage is not repaired and allowed to build up (Lord et al., 2012).

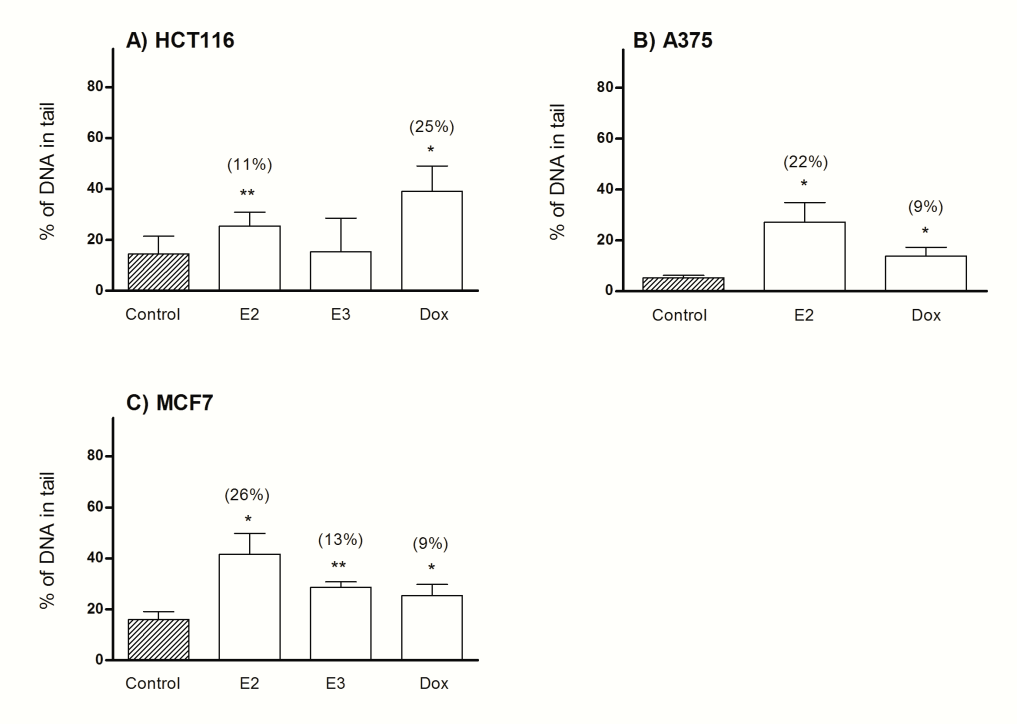

Figure 3. Effect of extract E2 and E3 on DNA damage in (A) HCT116, (B) A375 and (C) MCF7 cells evaluated by comet assay. Results are the mean+ SD at least three independent experiments. Significant differences (* $P \leq 0.05$ and ** $P \leq 0.01$) when compared with negative control were determined by one-way ANOVA, followed by the post-hoc Dunnett's test. Additionally, a Student's *t*-test was selectively used to access significant differences in HCT116 (E2), A375 (Dox) and MCF7 (Dox) in relation to the respective control.

In order to discern whether the anti-proliferative effect observed through the MTT reduction assay was due to the induction of cell death, nuclear condensation was analyzed after cells were subjected to a 48 h exposure to the respective IC_{50} values of extracts **E2** and **E3** (Figure 4). When analyzing nuclear condensation, a significant increase in the number of cell death was detected in most of the tested cell lines after treatment with extracts **E2** and **E3**. Extract **E3** exhibited a 19% increase cell death in HCT116 cells and a 10% increase in MCF7 cells in relation to the negative control. Extract **E2** presented a broader induction of cell death, having induced a significant increase of cell death in all tested cell lines. In colon carcinoma cell lines, extract **E2**

lead to an increment of 12% in HT29 cells and 17% in HCT116 cells regarding the negative control. Moreover, extract **E2** presented a 13% increase in A375 cells and 17% in MCF7 cells, in comparison to the negative control. Interestingly, extract **E2** showed a significantly higher induction of cell death than the positive control Dox in HCT116 and MCF7 cell lines. As referred before, some compounds isolated from extract **E2** and **E3** showed anti-proliferative effects however effects on cell death still unknown.

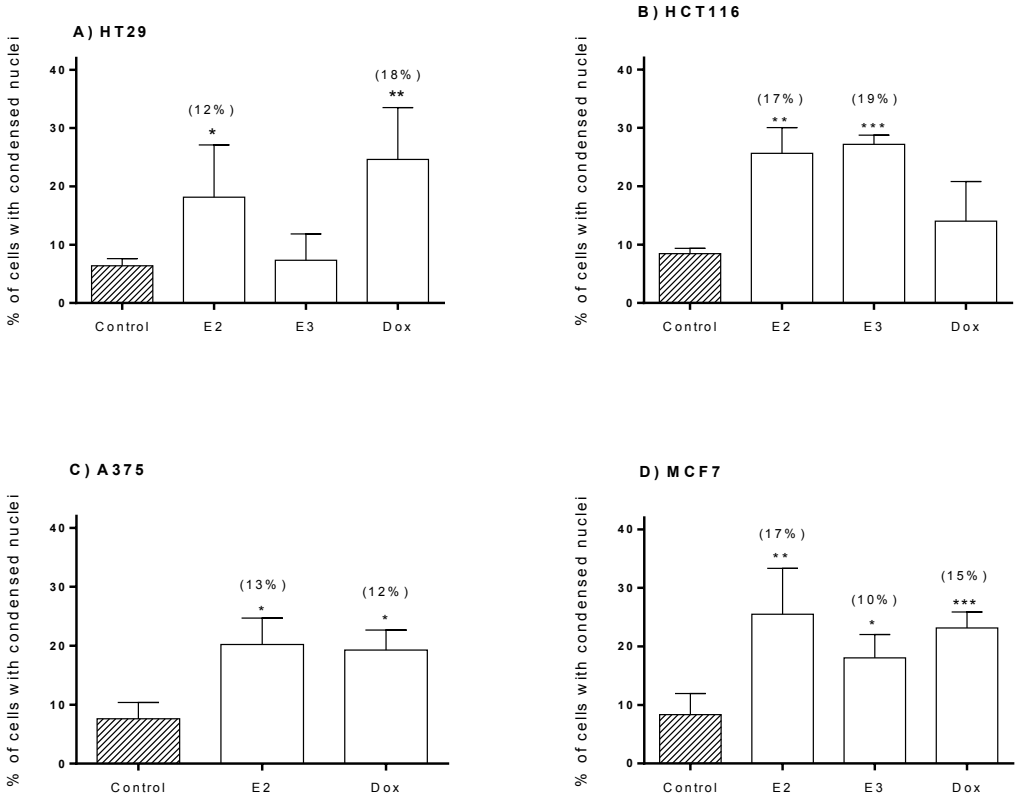

Figure 4. Effect of extract E2 and E3 on the induction of nuclear condensation in (**A**) HT29, (B) HCT116, (C) A375 and (D) MCF7 cells assessed by nuclear condensation assay. Percentages in brackets correspond to the increase in relation to negative control. Results are displayed as the mean +SD of at least three independent experiments. Significant differences (* P≤0.05; ** P≤0.01 and *** P≤0.001) when compared with negative control were determined by one-way ANOVA, followed by the post-hoc Dunnett's test. Additionally, a Student's *t*-test was selectively used to access significant differences in HT29 (E2) in relation to the control.

In summary, extract **E2** decreased cell proliferation in HT29, HCT116, A375 and MCF7 cells at both short and long-term, by decreasing the proliferative ability of a single cell to form a viable colony. Besides the anti-proliferative effects, extract **E2** also showed an induction of cell death in part due to the induction of DNA damage. Extract **E3** demonstrated an anti-proliferative effect at both short and long-term and cell death induction in HCT116 and MCF7 cells. However the mechanisms involved on induction of cell death seems to be in part different, since in HCT116 cells extract **E3** did not induce DNA damage. Extract **E3** also decreased proliferation in HT29 cells, however this decrease was only observed at short-term and without effect on cell death. These results show for the first time that *Neosartorya laciniosa* (KUFC 7896) and *Neosartorya fischeri* (KUFC 6344) crude ethyl extracts have anticancer activity in human colon carcinoma, breast adenocarcinoma and malignant melanoma cells by decreasing cell proliferation and increasing cell death. The investigation of transduction pathways and molecular targets involved on anticancer effects of extracts should be partaken in further studies. Also, this study supports the pertinence of further efforts for characterizing the exact bioactive compounds of the extracts that are involved in the effects we disclosed.

Acknowledgments

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Conflicts of Interest

There are no conflicts of interest to report.

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CHAPTER 3

MARINE-DERIVED FUNGI EXTRACTS AND ISOLATED COMPOUNDS ENHANCE THE ANTICANCER ACTIVITY OF DOXORUBICIN IN NON-SMALL CELL LUNG CANCER CELLS

This chapter is formatted according to the original manuscript to be submitted to the *Journal of Natural Products* or journals of adequate scope:

Castro-Carvalho B., Ramos A.A., Prata-Sena M., Dethoup T., Buttachon S., Kijjoa A. & Rocha E. (2014) Marine-derived fungi extracts and isolated compounds enhance the anticancer activity of doxorubicin in non-small cell lung cancer cells.

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3.1. Manuscript

Abstract: In view that drug resistance is a major concern in current chemotherapeutic approaches and that fungi (including of marine origin) are promising sources for bio-prospection of novel anticancer compounds, this study assessed firstly the *in vitro* anticancer activity of crude ethyl extracts of seven fungi — *Neosartorya tsunodae* (**E1**), *Neosartorya laciniosa* (**E2**), *Neosartorya fischeri* (**E3**), *Aspergillus similanensis* (**E4**), *Neosartorya paulistensis* (**E5**), *Talaromyces trachsypermus* (**E6**) and *Neosartorya siamensis* (**E7**) — when combined with doxorubicin, in a panel of seven cancer cell lines. Thenafter, the effect on cell viability of eight compounds isolated from extract **E7**, 2,4-dihydroxy-3-methylacetophenon (**C1**), nortryptoquivaline (**C2**), chevalone C (**C3**), tryptoquivaline H (**C4**), fiscalin A (**C5**), *epi*-fiscalin C (**C6**), *epi*-neofiscalin A (**C7**) and *epi*-fiscalin A (**C8**) was selectively evaluated in combination with doxorubicin in A549 lung cancer cells. Extracts **E1**, **E7** and **E2** demonstrated a significant enhancement of doxorubicin's cytotoxic activity, while also increasing cell death in A549 cells. All isolated compounds (except **C1**), potentiate the cytotoxicity of doxorubicin in A549 cells, some even more so than the originary extract. We concluded that the tested fungal extracts potentiate the anticancer action of doxorubicin, inhibiting cell proliferation and inducing cell death in A549 cells, while isolated compounds also enhance the activity of doxorubicin, validating the interest in these combinations. The data support that further studies are worth making, to characterize drug interactions and underlying mechanisms.

Keywords: Drug combinations; A549 cell line; *in vitro* anticancer activity; marine-derived fungi extracts; non-small cell lung carcinoma; *Neosartorya* sp.; *Aspergillus* sp.; *Talaromyces* sp.

Abbreviations: Doxorubicin (Dox); Dimethyl sulphoxide (DMSO); *Neosartorya tsunodae* KUFC 9213 (**E1**); *Neosartorya laciniosa* KUFC 7896 (**E2**); *Neosartorya fischeri* KUFC 6344 (**E3**); *Aspergillus similanensis* KUFA 0013 (**E4**); *Neosartorya paulistensis* KUFC 7894 (**E5**); *Talaromyces trachsypermus* KUFC 0021 (**E6**) and *Neosartorya siamensis* KUFA 0017 (**E7**); 2,4-Dihydroxy-3-methylacetophenon (**C1**); Nortryptoquivaline (**C2**); Chevalone C (**C3**); Tryptoquivaline H (C4); Fiscalin A (**C5**); *Epi*-fiscalin C (**C6**); *Epi*-neofiscalin A (**C7**); *Epi*-fiscalin A (**C8**).

1. Introduction

Lung cancer is a globally disseminated disease, having high mortality rates and an increment of more than a million new cases annually [1]. Non-small cell lung cancer (NSCLC) accounts for the great majority of lung cancer cases, yet there is still a lack of an efficient therapeutic protocol able to offer patients a better survivorship and an acceptable quality of life [2]. Also, drug resistance in lung cancer is a problem [3]. Indeed, this issue in lung and other cancer cells is an important concern for the medical and research community, as drug resistance is too often easily acquired by those cells during the administration of standard chemotherapeutic treatment [4]. Several mechanisms has been proposed in the acquisition of resistance, such as reduction of drug uptake, activation of drug detoxification, increase drug efflux and DNA repair capacity, and deflecting apoptotic pathway [5]. P-glycoprotein (P-gp) and multi-drug resistance-associated proteins (MRPs) are some of the best-known parts involved in drug resistance, and motivate tumor insensitivity to chemotherapeutic agents, where drugs are actively pumped from the cell to the outer membrane [6]. In the particular case of lung cancer, the lung resistance-related protein (LRP) is associated with lung cancer cell resistance to treatment with doxorubicin (Dox), consequently having an active role in the chemo-resistance of NSCLC to treatment [7]. A current approach to overcome this problem is the exploration of multi-drug combinations in an attempt to implement multi-target therapy as an alternative treatment, by affecting diverse cellular mechanisms implicated in cell resistance (e.g. drug uptake, drug metabolism, formation of DNA damage, DNA repair) and which can result in cell death [8]. This strategy has been increasingly implemented in diverse types of cancer [9,10].

The marine environment has proven to exert a selective pressure favorable to the production of novel and potent bioactive secondary metabolites by marine organisms [11]. Bio-prospection of marine-derived products is currently one of the main interests of pharmaceutical research, and several bioactive attributes have been found in those compounds, such as antibacterial, anti-diabetic, antifungal, anti-inflammatory, anti-protozoal, anti-tuberculosis, and antiviral activities [12]. Several products are currently under clinical trials, while others are already available as therapeutic agents for cancer patients (e.g. Citosar-U® and Yondelis®) [13].

Attending to the anticancer potential of marine products, we can logically reason that their combination with conventional anticancer drugs may constitute a strategy to overcome cancer drug resistance and mitigate some of the hazardous side effects associated to chemotherapy, possibly by decreasing the administrated dose of these

agents. Considering this rationale, the aim of our study was to assess whether the combination of the ethyl acetate extracts of six marine-derived fungi, namely *Neosartorya tsunodae* KUFC 9213 (**E1**), *Neosartorya laciniosa* KUFC 7896 (**E2**), *Neosartorya fischeri* KUFC 6344 (**E3**), *Aspergillus similanensis* KUFA 0013 (**E4**), *Neosartorya paulistensis* KUFC 7894 (**E5**), *Talaromyces trachsypermus* KUFC 0021 (**E6**) and *Neosartorya siamensis* KUFA 0017 (**E7**), enhanced the *in vitro* anti-cancer activity of doxorubicin on a panel of seven human cancer cell lines (HT29, HCT116, A375, A549, MCF7, U251 and HepG2 cells). Effects on cell viability and induction of DNA damage and nuclear condensation were evaluated. Furthermore, eight compounds isolated from *Neosartorya siamensis* KUFA 0017 (**E7**) — a) three quinazoline derivatives, nortryptoquivaline (**C2**), tryptoquivaline H (**C4**) and tryptoquivaline F (**C9**); b) four pyrazinoquinazoline derivatives, fiscalin A (**C5**), *epi*-fiscalin C (**C6**), *epi*-neofiscalin A (**C7**) and *epi*-fiscalin A (**C8**); c) the meroterpenoid chevalone C (**C3**); and d) 2,4-dihydroxy-3-methylacetophenon (**C1**) — were also evaluated for cell viability effect in combination with doxorubicin, in the A549 lung cancer cell line. With this assays we initiate exploring the hypothesis that because of their properties and anticancer potential these compounds may enhance doxorubicin's cytotoxic activity.

2. Materials and methods

2.1. *Reagents*

Doxorubicin (Dox), Dulbecco's Modified Eagle Medium (DMEM), Minimum Essential Medium Eagle medium (MEM), Roswell Park Memorial Institute medium (RPMI), McCoy's 5A Modified medium, penicillin/streptomycin solution, trypsin solution, 4,6-diamidino-2-phenylindole (DAPI) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO) was purchased from AMRESCO LLC (Solon, SO, USA). Fetal bovine serum (FBS) was purchased from Biochrom KG (Berlin, Germany). All other reagents and chemicals used were of analytical grade.

2.2. *Fungal material*

This study evaluated the effect of six marine-derived fungi, namely *Neosartorya tsunodae* (KUFC 9213) isolated from the marine sponge *Aka coralliphaga*, *Neosartorya siamensis* (KUFA 0017) isolated from a sea-fan (*Rumphella* sp.), *Neosartorya laciniosa* (KUFC 7896) isolated from a diseased coral (*P. lutea* ulcerative white spot), *Aspergillus similanensis* (KUFA 0013) isolated from the marine sponge *Rhabdermia* sp., *Neosartorya paulistensis* (KUFC 7897) isolated from the marine sponge *Chondrilla australiensis, Talaromyces trachsypermus* (KUFC 0021) isolated from the marine sponge *Clathria reianwardii*, and a soil-derived fungus, *Neosartorya fischeri* (KUFC 6344) isolated from coastal forest soil. The species were isolated and identified as described by Eamvijarn et al., (2013) and Gomes et al., (2014) [14, 15].

2.3. *Crude ethyl acetate extracts preparation*

Crude ethyl acetate extracts were prepared as previously described by Eamvijarn et al., 2013 and Gomes et al., 2014 [37,36]. Briefly, marine fungi were cultured in five 90 mm Petri dishes with malt extract agar (MEA) (for the strains KUFC 9213, KUFC 7896, KUFA 0013, KUFC 7897 and KUFA 0021) or with potato dextrose agar (for the strains KUFC 6344 and KUFA 0017) for one week. Then Erlenmeyer flasks containing rice and water were autoclaved, inoculated with the respective fungus and incubated for 30 days at 28º C. The mouldy rice was macerated in ethyl acetate, filtrated, the two layers were separated with a separatory funnel and the ethyl acetate solution was concentrated at a reduced pressure.

2.4. *Metabolite extraction*

The isolation of compounds from crude ethyl acetate of *Neosartorya siamensis* (KUFA 0017) was realized by Prof. Anake Kijjoa from ICBAS, as described in Buttachon et al., 2012, with some modifications [16]. The isolated compounds were: 2,4-dihydroxy-3-methylacetophenon (**C1**), nortryptoquivaline (**C2**), chevalone C (**C3**),

tryptoquivaline H (**C4**), fiscalin A (**C5**), *epi*-fiscalin C (**C6**), *epi*-neofiscalin A (**C7**) and *epi*-fiscalin A (**C8**).

2.5. *Cell culture*

A375 (malignant melanoma), U-251 (human glioblastoma), MCF7 (human breast adenocarcinoma) and A549 (non-small cell lung cancer) cell lines were purchased from ECACC. HepG2 cell line was generously offered by Prof. Rosário Martins from CIIMAR, Porto. HT29 and HCT116 (colon carcinoma) cell lines were provided by Prof. Carmen Jerónimo from IPO, Porto. All cell lines were maintained in a humidified atmosphere at 37ºC and 5% CO_2 in a cell culture incubator. Cell lines were maintained in MEM (HepG2, U251 and MCF7), DMEM (HT29, A375 and A549) and RPMI (HCT116). All mediums were supplemented with 1 % antibiotic solution (100 U/ml penicillin and 100 µg/ml streptomycin), 10 mM HEPES, 0.1 mM sodium pyruvate and 10% FBS. Medium was replaced every two days and tripsinization was performed once a week, or when cells reached a high confluence state (>80%).

Stock solutions of extracts, compounds and doxorubicin were dissolved in DMSO and stored at -20ºC. When initiating an experiment, extracts, compounds and doxorubicin were dissolved in fresh medium with a maximum concentration of 0.5% DMSO. Negative controls were prepared with fresh medium with 0.5% of DMSO.

2.6. *Evaluation of the effects of fungi extracts/compounds combined with doxorubicin on cell viability by MTT reduction assay*

To evaluate the effect of fungi extracts in combination with doxorubicin on cell viability, each cell line (HT29, HCT116, A375, A549, MCF7, U251 and HepG2 cells) was plated in 96-multiwell culture plates at densities ranging from $0.8x10^4$ to $1x10^4$ cells/ml (according to each cell line). Cells were incubated at 37ºC and 5% CO2 for 24 h until adhered. After adhesion, the medium was removed and new medium containing fungi extracts at 100 µg/ml alone or combined with Dox (IC_{50} of each cell line) was added to the cells. Cells incubated with Dox alone or with the vehicle solvent were used as a positive and negative control, respectively. All conditions contained medium with a maximum of 0.5% of DMSO.

To assess the effect of the compounds isolated from *Neosartorya siamensis* (KUFA 0017) on cell viability, A549 cells were plated in 96-multiwell culture plates at $0.8x10^4$ cells/ml. After cell adhesion, the medium was removed and cells were incubated with fresh medium containing the isolated compounds, 2,4-dihydroxy-3-methylacetophenon (**C1**), nortryptoquivaline (**C2**), chevalone C (**C3**), tryptoquivaline H (**C4**), fiscalin A (**C5**), *epi*-fiscalin C (**C6**), *epi*-neofiscalin A (**C7**), *epi*-fiscalin A (**C8**) at different concentrations (1, 10, 50 and 100 μM) alone or combined with Dox at 0.54 μM (IC_{50}). Cells were incubated for 48 h at 37ºC and 5% CO_2.

After 48 h of incubation with the extracts/compounds alone or combined with Dox, MTT to a final concentration of 0.5 mg/ml was added to the initial negative control (t = 0 h), and removed after 2 h incubation at 37ºC. The plate incubated for the remaining 46 h at 37ºC, 5% CO_2. At 48 h, MTT (0.5 mg/ml) was added to the remaining treatment conditions, and followed by 2 h incubation at 37ºC and 5% CO_2. To dissolve the formazan crystals, medium was removed, and 150 μl of organic solvent ethanol-DMSO (1:1) (v/v) was added to each well, and incubated for 10 min in a microplate shaker, protected from light. Optical density (O.D) was measured at a wavelength of 570 nm in a microplate reader (Multiskan EX, Labsystems, USA).

Cell viability (equation 1) was estimated by the cell ability to metabolize the MTT and was expressed as the percentage relative to negative control (cells treated with medium with 0.5% DMSO). The results correspond to the mean of at least three independent experiments; each one was carried out in duplicate. Cell growth during the experiment in the negative control were assessed by the increase of the number of cells in relation at t = 0 h. For the following assays, only the fungal extracts that decreased cells viability when in combination with Dox were used.

(Equation 1) Cell viability (%) = A sample x 100/ A control, where A corresponds to the optical density of sample at 570 nm.

2.7. *Evaluation of genotoxic effect of fungi extracts when combined with doxorubicin by comet assay*

A single cell alkaline electrophoresis assay (comet assay) was applied in order to assess whether the tested extracts/combinations caused DNA damage in the form of DNA single-strand breaks and alkali-labile sites. A549 cells were seeded at a density of 0.1 x 10^6 cells/ml in 24-well plates and incubated for 24 hours at 37ºC and 5% CO_2

until adhered. After adhesion, the medium was removed and new medium containing fungi extracts (100 and 200 µg/ml) and Dox (0.54 and 0.10 µM), either alone or in combination, was added to cells. After 24 and 48 h of incubation, medium was removed and cells washed with PBS and trypsinized. An amount of $5x10^4$ cells was collected from each condition and mixed with 100 µl of 0.5% (w/v) low melting point agarose and subsequently 70 µl of cells were set in microscope slides coated with 1% (w/v) of normal melting agarose. Slides were incubated overnight at 4ºC in a lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, pH 10) with the addition of 1% (v/v) Triton X-100. Prior to the electrophoresis, slides were incubated for 40 min at 4ºC in an alkaline electrophoresis buffer (300 mM NaOH, 1 mM Na_2EDTA, pH 13) for 40 min at 4ºC. The electrophoresis was performed in a horizontal electrophoresis chamber for 20 min (1V/cm). Samples were stained with DAPI (1 µg/ml) in the dark for 10 min before analysis by fluorescence microscopy (Olympus IX71). A minimum of 100 cells was scored per sample using CometScore® software (CometScore, TriTek Corp.) and percentage of tail intensity quantified.

2.8. *Evaluation of the effects on cell death induced by marine-derived fungi extracts when combined with doxorubicin through nuclear condensation assay*

The observation of cells presenting nuclear chromatin condensation was performed in order to assess the induction of cell death. A549 cells were seeded in the appropriate medium at a density of 0.1×10^6 cells/ml in 24-multiwell plates and incubated for 24 h at 37ºC and 5% CO_2 until adhered. After adhesion, the medium was removed and new medium containing fungi extracts (100 and 200 µg/ml) and Dox (0.1 and 0.54 µM), either alone or in combination, was added to the cells. Cells were incubated for 48 h at 37ºC and 5% CO_2. After the incubation, cells were washed, trypsinized and both non-adhered and adhered cells were collected. Samples were then centrifuged several times to isolate cells, which were then fixed with 4% paraformaldehyde (w/v) in PBS. The samples were mounted on poly-L-Lysine coated microscope slides using a Cell Spin Cytospin centrifuge (Thermo Scientific, USA) and stained with DAPI (1 µg/ml). Quantification of cells with condensed nuclei was attained by fluorescence microscopy (Olympus IX71). The percentage of cells with condensed nuclei was calculated from the ratio between cells with nuclear condensation and total number of cells. More than 300 cells were counted per sample.

2.9. *Statistical analysis*

The statistical analyses were performed with GraphPad Prism v6.0 software (GraphPad Software, La Jolla, CA, USA). Results were expressed as mean ± SD from at least 3 independent experiments. Outlier detection was performed using a ROUT test (Q=10%), as included in the cited software. Data were analysed for homogeneity of variances and normal distribution using the Bartlett's test and Kolmogorov-Smirnov test, respectively. A one-way ANOVA was performed to assess significant differences ($p \leq 0.05$) between treatment conditions and control, followed by *post hoc* Newman-Keuls multiple comparison test. An unpaired Student's *t*-test was used to verify significant differences, in selected pair comparisons of special interest.

3. Results

3.1. *Evaluation of fungi extracts effects on cell viability by the MTT colorimetric assay*

Alterations in cell viability of the cancer cell lines HepG2, HCT116, HT29, A549, A375, MCF-7 and U251 in response to a combinatory regimen of extracts (**E1**, **E2**, **E3**, **E4**, **E5**, **E6** and **E7**) + Dox were assessed by an MTT colorimetric assay. Extracts were tested at the concentration of 100 µg/ml in combination with Dox at IC_{50} for each cell line, and then represented as (100/0.54) [1] in the case of A549 cells. Attending that the values of the IC_{50} of these extracts alone range from 124 µg/ml to > 200 µg/ml (previous data), a lower concentration of extracts was used (100 µg/ml) in combination with Dox. The negative control exhibited significant cell growth over a period of 48 h for all cell lines demonstrating that the cells were in the exponential growth phase (data not shown).

Only three extracts (**E1**, **E2** and **E7**) in A549 lung cancer cell line presented statistically relevant ($p < 0.05$) results regarding the effect on cell viability when combined with Dox in comparison with cells treated with Dox alone. None of the remaining fungi extracts combined with Dox in A549 and other cell lines exhibited a statistically significant decrease in cell viability when compared with Dox alone (data not shown). Results from the cell viability of A549 cell line (Figure 1) are expressed as the per-

[1] From this point on, the notation, e.g. E+Dox (100/0.54), refers to extract at 100 µg/ml and Dox at 0.54 µM in A549 cells. Combinations with compounds and doxorubicin, e.g. C+Dox (100/0.54,) refer to compound at concentrations in µM and Dox in concentrations in µM.

centage in relation to the negative control. Dox alone (0.54 µM) exhibited a marked effect in cell viability, representing a 40% decrease in cell viability in relation to the negative control. Also, extract **E7** presented a significant decrease in cell viability, by 21%, when compared with the negative control. Extracts **E1** and **E2** at 100 µg/ml, exhibited a slight decrease in cell viability although not statistically significant when compared to the negative control. The combinatory regimens of extract **E1**+Dox (100/0.54); extract **E7**+Dox (100/0.54) and extract **E2**+Dox (100/0.54) manifested a significant cell viability inhibition of 26%, 22% and 26%, respectively, when compared with the positive control.

Subsequent assays were performed only with extract **E1**, **E2** and **E7** in the A549 lung cancer cell line, as a consequence of the results obtained in the screening test by the MTT assay, which demonstrated that the other extracts combined with Dox did not increase the cytotoxic effect of Dox in any of the cell lines tested.

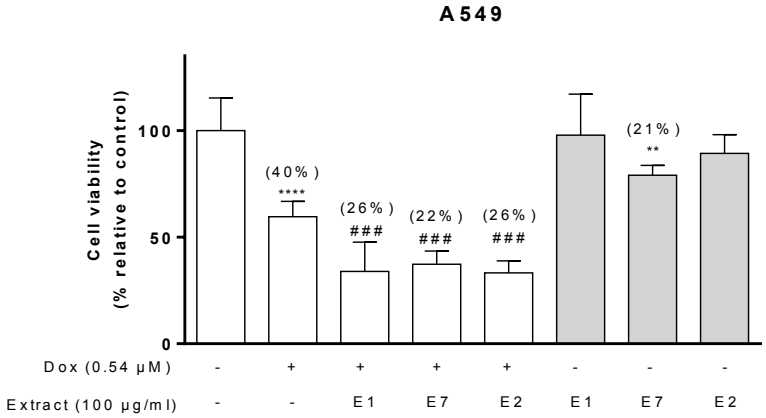

Figure 1 – Effect of extracts **E1**, **E2**, **E7** at 100 µg/ml alone and in combination with Dox at 0.54 µM on cell viability of the A549 lung cancer cell line. Percentages in brackets refer to a decrease in cell viability in relation to the negative control (medium with 0.5% DMSO) or positive control (Dox at 0.54 µM). Results are the mean + SD of at least four independent experiments. Significant differences (** $p \leq 0.01$, *** $p \leq 0.001$) when compared with the negative control and (## $p \leq 0.001$) with the positive control (Dox alone) were determined by a one-way ANOVA followed by the *post-hoc* Newman-Keuls multiple comparison test.

3.2. *Effects on DNA damage induction by marine fungi extracts combined with Dox*

The comet assay was used to evaluate if the combination of extracts and Dox has genotoxic activity, by inducing DNA damage (via DNA strand breaks and alkali-labile

sites) in the A549 lung cancer cell line after 24 h and 48 h of treatment. Results (% of DNA in tail) were expressed in percentage relative to the negative control, reflecting the increase of DNA damage in comparison to control. At 24 h, no genotoxicity was observed (data not shown). However, significant results were observed with a 48 h exposure time (Figure 2). Dox (0.10 and 0.54 μM) alone was not able to induce a significant increase of DNA damage assessed by comet assay. Also, none of the treatments in which **E1** was used showed any statistically relevant increase of DNA damage relative to the use of Dox alone (Figure 2A). In the case of extract **E7**, only the combination of **E7**+Dox (200/0.54) exhibited a significant increase of 12% in DNA damage, in comparison to the negative control, and 10% relative to Dox at 0.54 μM alone (Figure 2B). None of the remaining results had statistical relevance when compared with the respective control. Figure 2C shows that **E2** alone, at 200 μg/ml, significantly increases DNA damage in 9% relative to the negative control. Increase of DNA damage in comparison to the positive control was observed in **E2**+Dox. The combinations of **E2**+Dox (200/0.10) and **E2**+Dox (200/0.54) induced an increase of 5% and 9% in comparison with Dox at 0.1 μM and 0.54 μM, respectively.

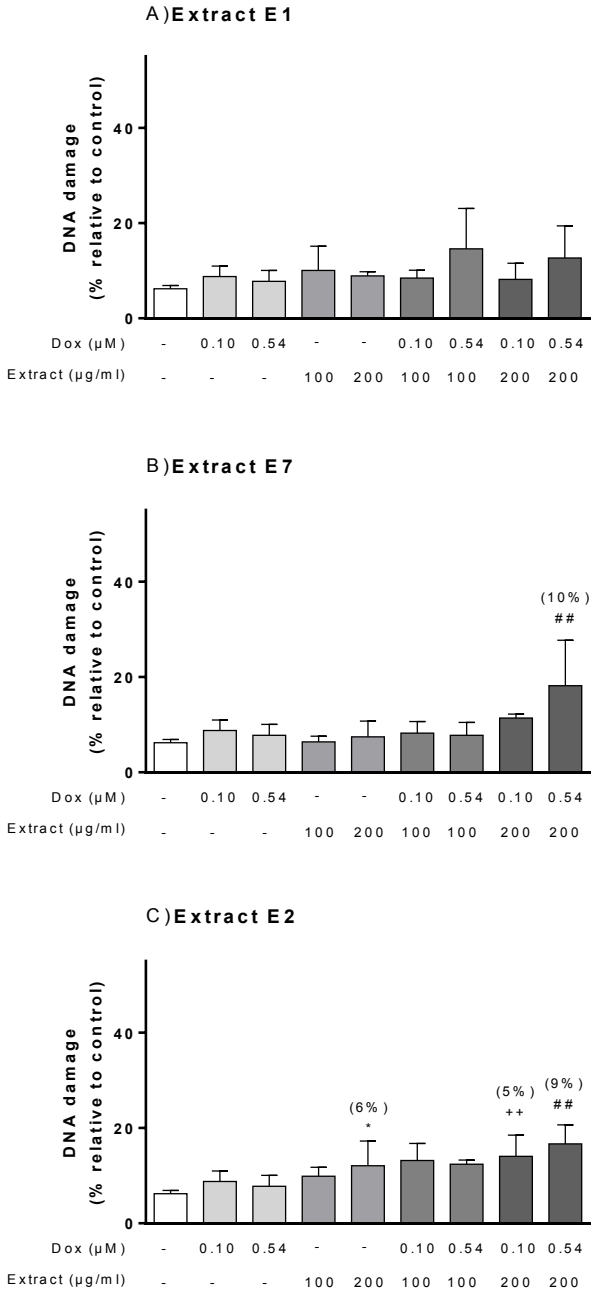

Figure 2 – Effect of extracts **E1** (A), **E7** (B), **E2** (C) at 100 µg/ml and 200 µg/ml alone and in combination with Dox at 0.1 µM and 0.54 µM on the induction of DNA damage in A549 cell line assessed by comet assay. Percentages in brackets refer to the increase of DNA damage in relation to the negative control (medium with 0.5% DMSO) or positive control (Dox at 0.1 µM or 0.54 µM). Results are the mean + SD of at least three independent experiments. Significant differences (*$p \leq 0.05$) when compared with the negative control, and (## $p \leq 0.01$) when compared with Dox at 0.54 µM, were determined by a one-way ANOVA followed by the *post-hoc* Newman-Keuls multiple comparison test. Additionally, an unpaired Student's *t*-test was used to determine significant differences (++ $p \leq 0.01$) between extract **E2**+Dox (200/0.10) at in relation to the positive control (Dox at 0.10 µM).

3.3. *Assessment of cell death induced by marine fungi extracts in combination with doxorubicin by a nuclear condensation assay*

Nuclear chromatin condensation was assessed in the A549 cell line after 48 h exposure to the different combination regimens, as described in material and methods section. As shown in Figure 3, the positive controls for this experiment, Dox at 0.10 μM and 0.54 μM induced a significant increase in the percentage of cells with nuclear condensation by 6% and 8%, respectively, when compared with the negative control (cells incubated with medium and 0.5% DMSO). Regarding the combinations with extract **E1**, both the **E1**+Dox (100/0.54) and **E1**+Dox (200/0.54) exhibited a statistically significant increase of 19% and 22%, respectively, when comparing with Dox 0.54 μM alone (Figure 3a). Combinations with extract **E7**, namely **E7**+Dox (100/0.54) and **E7**+Dox (200/0.54) were also able to induce a significant increase in cells presenting nuclear condensation, by exhibiting a 8% and 23% increase, respectively, in comparison with Dox 0.54 μM (Figure 3B). The combination of Dox with extract **E2** allowed the observation of a greater induction of cells with nuclear condensation when compared to the other extracts (**E1** and **E7**) in combination with Dox. The combination of **E2**+Dox (200/0.54) caused an increase of 28% in relation to Dox 0.54 μM alone. The combination of **E2**+Dox (100/0.54) exhibited an increase of 10% in comparison with Dox 0.54 μM alone (Figure 3C). None of the results regarding extracts **E1**, **E7** or **E2** at 100 μg/ml or 200 μg/ml combined with Dox at 0.10 μM were able to induce a significant increase in the number of cells with nuclear condensation when compared with Dox 0.10 μM alone.

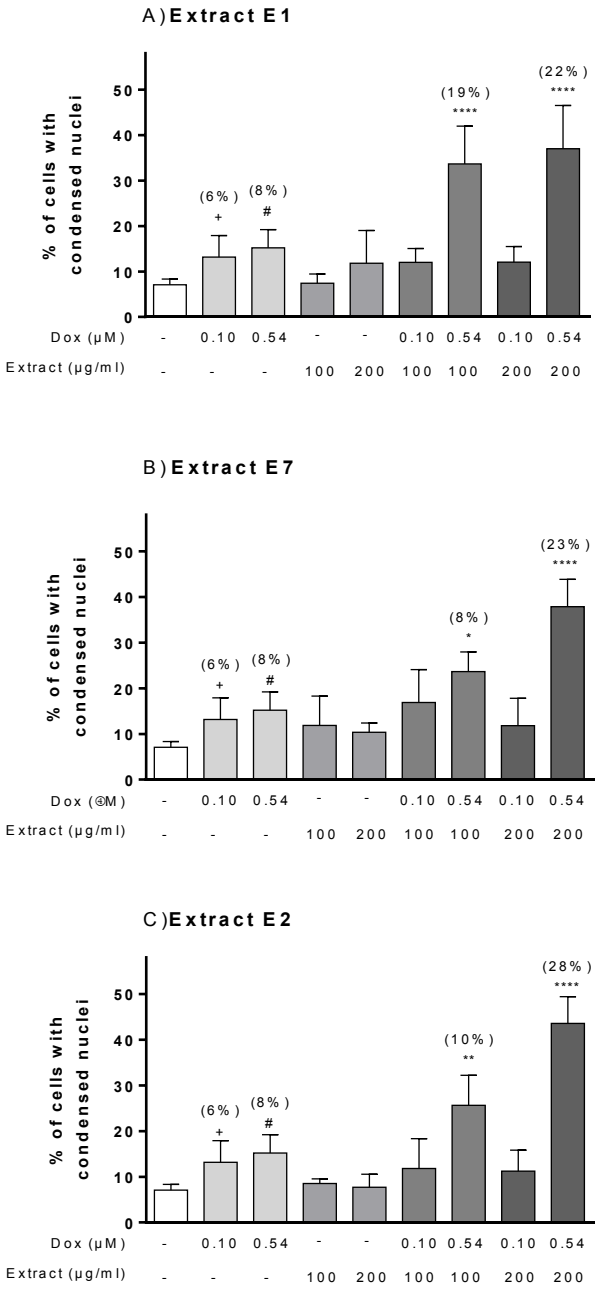

Figure 3 – Effect of extracts **E1** (A), **E7** (B), **E2** (C) at 100 µg/ml and 200 µg/ml alone and in combination with Dox at 0.1 µM and 0.54 µM on the induction of nuclear condensation in A549 cell line. Percentages in brackets refer to the increase in cells with condensed nuclei in relation to the negative control (medium with 0.5% DMSO) or positive control (Dox at 0.1 µM or 0.54 µM), respectively. Results are the mean + SD of at least three independent experiments. Significant differences (# $p \leq 0.05$) when compared with the negative control and (*$p \leq 0.05$; ** $p \leq 0.01$ and *** $p \leq 0.001$) when compared with the respective positive control, were determined by a one-way ANOVA followed by the *post-hoc* Newman-Keuls multiple comparison test. Additionally, an Student's *t*-test was used to determine significant differences (+ $p \leq 0.05$) between Dox at 0.10 µM in relation to the negative control.

3.4. *Evaluation of the effect of eight compounds isolated from* Neosartorya siamensis *in combination with doxorubicin on A549 cell viability by an MTT reduction assay*

The A549 cells were exposed to the isolated compounds (1, 10, 50 and 100 μM) alone or with Dox 0.54 μM (IC_{50}) for 48 h. An MTT assay was made to assess the effect on cell viability of these compounds alone and in combination with Dox; only significant ($p < 0.05$) decreases in cell viability in relation to Dox will be presented.

As shown in Figure 4, Dox at 0.54 μM significantly decreased cell viability by 45 % in relation to the negative control. The cytotoxic effect of Dox increased when combined with compounds, except in the case of 2,4-dihydroxy-3-methylacetophenone (**C1**) that did not demonstrate a significant decrease in cell viability when compared with Dox 0.54 μM alone. However, compound **C1**, when used alone at 1 μM, demonstrated to decrease cell viability by 32% in comparison with the negative control (Figure 4A). Nortryptoquivaline (**C2**) combined with Dox 0.54 μM enhanced the cytotoxic effect by 32% (**C2**+Dox (50/0.54)) and 42% (**C2**+Dox (100/0.54)) in comparison to Dox 0.54 μM alone. Nortryptoquivaline (**C2**), when used alone at 1, 10, 50 and 100 μM, demonstrated to decrease cell viability by 22%, 14%, 22% and 43%, respectively, in comparison with the negative control (Figure 4B). Chevalone C (**C3**) combined with Dox 0.54 μM increased the effect by 14% (**C3**+Dox (50/0.54)) and 24% (**C3**+Dox (100/0.54)) in comparison to Dox 0.54 μM alone; none of the remaining combinations of **C3** and Dox expressed a significant decrease in cell viability. Chevalone C (**C3**), when used alone at 50 μM, and 100 μM demonstrated to decrease cell viability in 25%, 44%, respectively, in comparison with the negative control (Figure 4C). Tryptoquivaline H (**C4**), combined with Dox 0.54 μM significantly increased the effect of Dox 0.54 μM by 22% in (**C4**+Dox (50/0.54)) and 30% in (**C4**+Dox (100/0.54)); none of the remaining combinations of **C4** and Dox expressed a significant decrease in cell viability. Compound **C4**, when used alone at 50 μM and 100 μM, demonstrated ability to decrease cell viability by 29% and 30%, respectively, in comparison with the negative control (Figure 4D). Fiscalin A (**C5**) combined with Dox 0.54 μM enhanced the effect by 20% (**C5**+Dox (50/0.54)) and 31% (**C5**+Dox (100/0.54)) in comparison to Dox 0.54 μM alone. The remaining combinations of **C5** and Dox did not express a significant decrease in cell viability. A549 cells treated alone with compound **C5** at 100 μM demonstrated a decrease on cell viability of 38%, in comparison with the negative control (Figure 4E). *Epi*-fiscalin C (**C6**) combined with Dox 0.54 μM enhanced the effect of Dox by 20% (**C6**+Dox (50/0.54)) and 31% (**C6**+Dox (100/0.54)). None of the remaining combinations of compound **C6** and Dox expressed a signifi-

cant decrease in cell viability. *Epi*-fiscalin C (**C6**), when used alone at 50 µM and 100 µM, decreased cell viability by 34% and 41% respectively, in comparison with the negative control (Figure 4F). *Epi*-neofiscalin A (**C7**) combined with Dox 0.54 µM significantly enhanced the cytotoxic effect by 24% (**C7**+Dox (50/0.54)), and 34% (**C7**+Dox (100/0.54)) in comparison to Dox 0.54 µM alone. When used alone, compound **C7** was unable to induce a significantly decrease in cell viability in comparison with the negative control in any of the tested concentrations (Figure 4G). *Epi*-fiscalin A (**C8**) combined with Dox 0.54 µM significantly increased the effect of Dox by 20% (**C8**+Dox (50/0.54) and 23% (**C8**+Dox (100/0.54)). None of the remaining combinations and **C8** alone exhibited a significant decrease of cell viability when compared with the respective controls (Figure 4H).

Table 1 – Half maximal inhibitory concentrations (IC_{50}) and respective 95% confidence intervals of compounds **C1** to **C8** regarding the A549 lung cancer cell line.

	A549 cell line	
Compounds	**IC_{50} µM**	*95% confidence interval*
C1	215.0	(53.5 – 869.6)
C2	123.8	(83.3 – 184.1)
C3	118.9	(85.8 – 164.7)
C4	144.8	(74.0– 272.3)
C5	135.1	(81.8 – 223.1)
C6	199.2	(91.8 – 432.0)
C7	188.3	(103.5 – 342.4)
C8	557.0	N.D

N.D – not determined

Table 1 demonstrates the half maximal inhibitory concentrations (IC_{50}) of the compounds **C1** to **C8**. Chevalone C (**C3**) exhibited the lowest IC_{50} value (118.9 µM) of all compounds. Followed by Nortryptoquivaline (**C2**), Fiscalin A (**C5**), Tryptoquivaline H (**C4**), *Epi*-neofiscalin A (**C7**), *Epi*-fiscalin C (**C6**) and 2,4-Dihydroxy-3-methylacetophenon (**C1**) with an IC_{50} of 123.8 µM, 135.1 µM, 188.3 µM, 144.8 µM and 199.2 µM, respectively. *Epi*-fiscalin A (**C8**) demonstrated the highest IC_{50} of all compounds (557.0 µM).

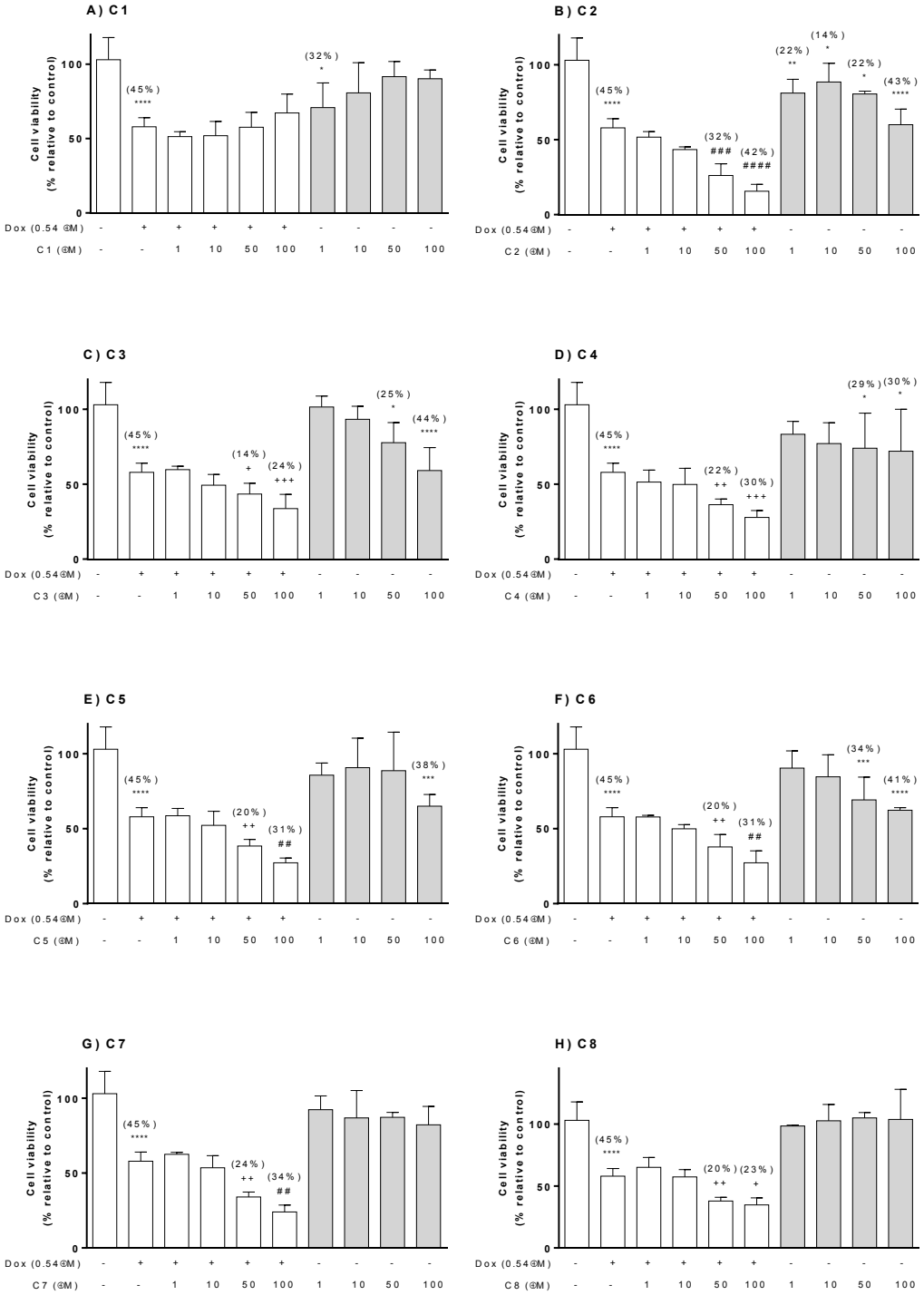

Figure 4 – Effect of compounds **C1** (A)**, C2** (B)**, C3** (C)**, C4** (D)**, C5** (E)**, C6** (F)**, C7** (G)**, C8** (H) at 1, 10, 50 and 100 µM alone and in combination with Dox at 0.54 µM on cell viability in the A549 cell line. Percentages in brackets refer to a decrease of cell viability in relation to the negative control (medium with 0.5% DMSO) or positive control (Dox at 0.54 µM), respectively. Results are expressed as mean + SD of at least 3 independent experiments or at least 2 in the case of **C7** and **C8** in combination with Dox at 0.54 µM. Significant differences (*$p \leq 0.05$; ** $p \leq 0.01$ and *** $p \leq 0.001$) when compared with the negative control and (## $p \leq 0.01$ and

$p \leq 0.001$) when compared with the positive control were determined by one-way ANOVA followed by the *post-hoc* Newman-Keuls multiple comparison test. Additionally, significant differences (+ $p \leq 0.05$, ++ $p \leq 0.01$, +++ $p \leq 0.001$) when compared with the positive control (Dox) were determined by an unpaired Student's *t*-test.

3.5. *Comparison between the cytotoxic effect of* Neosartorya siamensis *extract and the isolated compounds when in combination with doxorubicin*

The comparison of the cytotoxic effect of extract **E7** in combination with Dox and the compounds (isolated from this extracts) in combination with Dox is represented in the Figure 5. The analysis was performed with the concentrations of the compounds in combination that demonstrated a cytotoxic effect significantly higher than Dox alone (Figure 4), with the intent to comprehend what is the cytotoxic contribute of each compound in the effect of the extract of origin (**E7**) combined with Dox.

We observed that the combination of **C2**+Dox (100/0.54) was the most notorious result, demonstrating a 22% decrease in cell viability when compared with extract **E2**+Dox (100/0.54). Also, **C2**+Dox (50/0.54), **C5**+Dox (100/0.54) and **C7**+Dox (100/0.54) exhibited a significant decrease in cell viability by 11%, 10% and 13%, respectively when compared with **E7**+Dox (100/0.54). None of the remaining combinations revealed significant results (Figure 5).

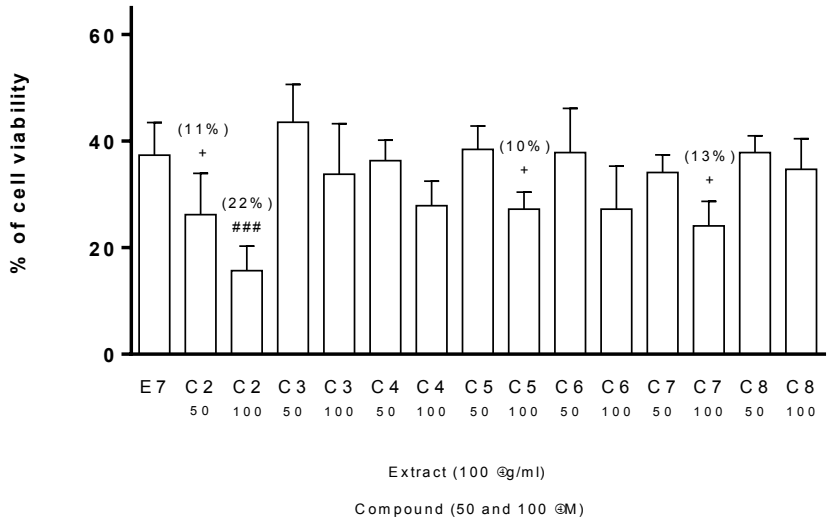

Figure 5 – Comparison between the effect of **E7** (100 µg/ml) and the isolated compounds **C2, C3, C4, C5, C6, C7** and **C8** at 50 µM and 100 µM in combination with Dox at 0.54 µM in the A549 cell line. Percentages in brackets refer to a decrease of cell viability in relation to the extract (**E7** at 100 µg/ml + Dox at 0.54 µM). Results are expressed as mean + SD at least three independent experiments or at least two in the case of **C7** and **C8** in combination with Dox at 0.54 µM Significant differences (### $p \leq 0.001$) when compared with the extract were determined by a one-way ANOVA followed by the post-hoc Newman-Keuls multiple comparison test. Additionally, significant differences (+ $p \leq 0.05$ and) when compared with extract were determined by an unpaired Student's *t*-test.

4. Discussion

During chemotherapy, cancer cells frequently acquire drug resistance, limiting the efficiency of treatment and therapeutical choices for the patient [17]. Doxorubicin is an anticancer drug used in the treatment of several types of cancer [18]. Apart from several side effects such as cardiac toxicity, drug resistance is a frequent situation when doxorubicin therapy is implemented [19]. A strategy to overcome such problems relies on the implementation of multi-drug combination therapies, which could lessen adverse side effects and potentiate the chemotherapeutic drug's effect [19].

In our study we demonstrated, for the first time, that the *in vitro* anticancer activity of doxorubicin could be enhanced by combination with marine-derived fungi extracts in lung cancer cells. We also identified some isolated compounds from extract **E7** that could be involved in the potentiation of the cytotoxic effect of doxorubicin. In this study, the effect in cell viability of seven crude ethyl extracts derived from *Neosar-*

torya tsunodae (**E1**)*, Neosartorya laciniosa* (**E2**)*, Neosartorya fischeri* (**E3**)*, Aspergillus similanensis* (**E4**)*, Neosartorya paulistensis* (**E5***), Talaromyces trachsypermus* (**E6**) and *Neosartorya siamensis* (**E7**) in combination with Dox, was assessed in a panel of seven cancer cell lines (HepG2, HCT116, HT29, A549, A375, MCF-7 and U251). From the array of extracts and cell lines tested, only extracts **E1**, **E7** and **E2** combined with Dox in A549 cell line showed a significant decrease in cell viability when compared with the use of Dox alone. Dox exhibited a potent decrease in cell viability in all cell lines. However, the extracts alone possessed low to moderate effect regarding the decrease of cell viability, with IC_{50} values ranging from 124 µg/ml to > 200 µg/ml µM (data not shown). In A549 cells, only extract **E7** alone presented a significant decrease in cell viability when compared with the respective control. Interestingly, the extracts that combined with Dox exhibited an enhancement of the decrease in cell viability were not those that presented the most appreciable effect in cell viability when they are tested alone. Extracts with no significant effect on cell viability, namely **E1** and **E2**, when combined with Dox, considerably enhanced the effect of Dox alone by 26% and 27%, respectively (Figure 1). While, extract **E7** showed a moderate effect in decreasing cell viability (25%) when used alone, and in combination it also enhanced the effect of Dox by 23%. It is possible that extracts **E1** and **E2** may be acting to some extent in a mechanism that interferes with doxorubicin's activity, while extract **E7** may act by independent and distinct mechanism of Dox. The isolation of compounds from these extracts seems to be of relevance in order to precise which compounds are responsible for the demonstrated effects.

To evaluate possible mechanisms involved on the enhancement of cytotoxic activity of Dox when in combination with extracts, effects on cell death and DNA damage were assessed. Apoptosis is characterized by several hallmarks such as cell shrinkage, retraction of pseudopods, rounding of the cell, reduction of cellular volume, nuclear fragmentation and chromatin condensation. Nuclear condensation is a feature that is common to both mitosis and cell death, however, chromatin condensation is much more intense in the latter case, and ultimately culminates in nuclear fragmentation [20,21]. Induction of cell death by assessing nuclear chromatin condensation was observed after 48 h exposure to treatments. None of the extracts alone (**E1**, **E7** and **E2**) at tested concentrations was able to induce an increase in the number of cells with condensed nuclei. This suggests that the extracts alone do not have ability to induce cell death. In fact, Dox exhibited an increase in cells with condensed nuclei in relation to the negative control. All three extracts combined with Dox (0.54 µM)

greatly increased the number of cells with condensed nuclei in comparison to Dox (0.54 μM) alone. All combinatory regimens demonstrated the same behavior, by increasing in the number of cells with condensed nuclei when shifting the concentrations from 100 μg/ml to 200 μg/ml of extracts. However the effect on cell viability with extracts at 200 μg/ml + Dox at 0.54 μM are not know and should be addressed. When extracts are in combination with a low dose of Dox (0.10 μM) none significant increase in the nuclear condensation was observed. Several mechanisms of action have been proposed for the use of Dox, including DNA intercalation, DNA binding and alkylation, DNA cross-linking, interference with helicase activity, inhibition topoisomerase II and generation of reactive oxygen species (ROS) [18]. Some studies report dose-dependent effect with the use of Dox, suggesting that different mechanism of actions may occur according with the dose of Dox used [22]. Our results show that the induction of cell death is dependent of the concentration of Dox, with a minimal concentration of Dox below of which no effect is observed independently of extract concentration. Since no effect was detected from the use of the extracts alone, these observations suggest that the underlying mechanism of cell death is tightly associated to a higher dose of Dox, which may trigger a different mechanism than when exposing cells to a lower dose of Dox. Additionally, when the higher concentration of Dox is combined with the extracts the induction of cell death is greatly augmented.

Several anticancer drugs act by inducing a high level of DNA damage and if the repair ability is overcome then induction of apoptotic pathways may be activated and in this way eliminating tumors cells. So, induction of DNA damage is often approached to predict cell death mediated by genotoxic drugs, whose action involves the disruption of nuclear DNA [23]. Cell death may indeed occur if the damage occurs in intense way and in fundamental regions of the DNA, with no repair mechanism to lessen their consequence. In this sense, the use of an alkaline comet assay, that allows the detection of DNA strand breaks (SB) and alkali-labile sites, could provide evidence of an early interaction of a chemical agent and the nuclear DNA [24,25]. Single-strand breaks (SSB) and double-strand breaks (DSB), are types of DNA damage caused by certain anticancer drugs such as Dox [26].

In our study, the combination of **E7**+Dox (200/0.54) and **E2**+Dox (200/0.54) after 48 h, demonstrated a significant increment of DNA damage when compared with Dox alone. The extract **E2** alone at a concentration of 200 μg/ml was the only extract that was able induce an increase of 6% in DNA damage by its own, in relation to the

negative control, but without effect on cell death, maybe because this damage is not sufficient to activate the cell death pathways. Extracts **E7** and **E2** when combined with Dox increase DNA damage detected by comet assay. This could be due a decrease of DNA repair capacity and/or a decrease of antioxidant defenses with accumulation of DNA damage. Some studies showed that Dox induce DNA damage, mainly DSBs, due to it interaction with topoisomerase II and by production of reactive oxygen species (ROS) [27]. This damage will activate cell cycle arrest and DNA repair but if they get accumulated apoptosis may be initiated [28]. In our study, none of the concentrations of Dox alone were able to induce significant DNA damage in A549 cell line. This reinforces the issue of drug resistance mechanisms in lung cancer here represented by A549 non-small cell lung cancer line [7,3]. In addition, none of the combinatory regimens that enhanced cell viability decrease (**E**+Dox (100/0.54)) induced significant DNA damage when compared with the positive control (Dox at 0.54 μM). This could suggest that the underlying mechanism associated to the decrease of cell viability of these combinations at low concentrations of extracts does not rely upon induction of DNA damage (strand breaks). However, for other combinations induction of DNA damage may occur. For a better comprehension of the kinetics of DNA damage, a further exposure should be implemented in the attempt to determine if DNA damage is increasing or decreasing over time and concentration.

In the case of extracts **E1** and **E2**, other mechanisms may be involved, for instance, the inactivation of drug transporters (P-gp, LRP, MDR) by allowing the intracellular accumulation of Dox. These hypothesis need confirmation, nonetheless seem well corroborated by the total lack of toxicity associated with the use of the extracts alone, much like the effect demonstrated by the use of a pump inhibitor alone, such as verapamil, in cancer cell lines that when used in combination with toxic drugs, the drug's effect is greatly potentiated [29]. In complement, an activation of the p53 tumor suppressor gene by these extracts is also plausible, due to the involvement of p53 on the cytotoxic effect of Dox, namely in A549 cells [30,31]. When in combination with Dox, and in a scenario where the p53 gene is functional, cells will experience higher rates of cell death, since p53 has an active role in the potentiation of pro-apoptotic pathways [31].

When looking for studies that searched for potential enhancements of the effects of established chemotherapeutics by first combining them with extracts, we found that an extract from the fucoid algae (200 μg/ml to 400 μg/ml) *Cladosiphon navae-caledoniae* was tested in combination with cisplatin (5 μM to 10 μM), tamoxifen (10

μM to 20 μM) and paclitaxel (2.5 nM to 5 nM) in two breast cancer cell lines (MDA-MB-231 and MCF-7) [32]. The authors obtained around 80% of cell growth inhibition for both cell lines and reported the occurrence of nuclear condensation from 52% to 66%. Despite these assays and the data cannot be compared directly to our scenario, the fact is that herein the decrease in cell viability was around 66%, while using a concentration 4-fold lower of the extract and 18 to 37-fold lower concentration of the (Dox) chemotherapeutic agent (this when compared to cisplatin and tamoxifen). Regarding the induction of nuclear condensation, we reported an increase of 23% to 28%, by using a concentration 9 to 19-fold lower of our chemotherapeutic agent (in comparison to cisplatin and tamoxifen). In spite of our results regarding anticancer activity being slightly less enhanced than the latter study, our results point towards an equal trend, suggesting that the extract hold compounds that have a potential to be associated with Dox for gains in efficacy at lower doses.

The overall enhancement of the cytotoxicity of Dox when combined with extracts occurs by induction of apoptosis and in some experimental conditions with the induction of DNA damage. However, other mechanisms such as drug transport, drug metabolism and DNA repair could be involved on the potentiating of cytotoxicity activity of Dox in A549 cells and further studies should be equated to address these points.

Taking in mind the effect of extract **E7** on the enhancement of the cytotoxic activity of Dox in A549 cells, there was a heightened interest in analyzing the eight compounds isolated from this same crude ethyl extract in combination with Dox, in an attempt to pinpoint the compound or compounds responsible for the effect observed. For this, the eight compounds isolated from the extract **E7** were tested alone and in combination with Dox and the effect on cell viability was evaluated. The combinatory regimens of compounds and doxorubicin demonstrated better results in terms of decreasing cell viability, when compared with the use of the compounds alone. Combinatory regimens with Dox at 0.54 μM and the compounds chevalone C (**C3**), tryptoquivaline H (**C4**), fiscalin A (**C5**), *epi*-fiscalin C (**C6**), *epi*-neofiscalin A (**C7**) and *epi*-fiscalin A (**C8**) demonstrated a very similar effect amongst themselves, while nortryptoquivaline (**C2**) demonstrated the most prominent effect of all combinations. Overall, compound combinations ranged from no effect to 42% more effective than Dox alone. Several experiments have tried to formulate drugs based in the combination of commercialized drugs or novel compounds with doxorubicin [33,34]. Our results suggest that some of our compounds could be explored with the same purpose.

When used alone, some compounds did not exhibit significant results in comparison to the positive control, such as **C7** and **C8**. The other compounds tested, when alone, were able to decrease cell viability when compared with negative control. In some cases, such as compounds **C2, C3**, **C4**, **C5** and **C6**, there was a great effect, with a decrease in cell viability similar to that observed with Dox at 0.54 µM alone.

2,4-Dihydroxy-3-methylacetophenone (**C1**) alone at lowest tested concentration (1 µM), exhibited a highly significant effect decrease in cell viability when compared with the negative control. None of the higher concentrations showed any considerable effect, neither did the combination of the compound with Dox (0.54 µM) present any additional effect in relation to Dox alone.

In the study by Buttachon et al., (2012), some of the compounds common to those tested in our study were isolated from a terrestrial strain of *Neosartorya siamensis* (KUFC 6349), namely, compounds **C1**, **C4**, **C5**, **C7** and **C8**. Anti-proliferative activity of compounds **C4** and **C8** was assessed in A549 and four other cell lines [16]. The IC_{50} values were reported as being > 100 µM for A549 cell line, which are consistent with our own results (Table 1.), replicating and reinforcing the compounds' potential.

Additionally, in a study by Sogngam et al., (2014), four compounds that are common to our compounds **C3**, **C5**, **C6** and **C8** were isolated from the fungus *Xylaria humosa* and their cytotoxic activity was tested in NCI-H187 (small-cell lung cancer) and two other cancer cell lines. Compound **C3** exhibited a great effect with an IC_{50} value of 17.7 µg/ml, while none of the other compounds exhibited an appreciable effect [35]. In our study, compound **C3** exhibited an IC_{50} value of 53.8 µg/ml. The IC_{50} value of Dox in this cell line was of 0.11 µM. The A549 cell line demonstrated a much higher resistance both when exposed to compound **C3** and Dox when compared with NCI-H187. This may perhaps be explained by differing genotypic profiles between the cells. [36, 37].

In fact, three of the tested compounds (**C2**, **C5** and **C7**) when in combination with Dox exhibited a significant decrease in cell viability greater than the extract **E7** in combination with Dox. Compound **C2** was the most effective by decreasing cell viability 22% more than the extract **E7**. The remaining two compounds (**C5** and **C7**) showed a similar effect by decreasing cell viability in 10 and 13%, respectively, when comparing to **E7** in combination. These results suggest that compounds **C2**, **C5** and **C7** could be main players in the effect on cell viability shown by the extract **E7**. None-

theless, it was only possible to observe appreciable effects in cell viability reduction at higher concentrations (50 μM and 100 μM), with the exception of **C1**. Compound concentrations of 100 μM are equivalent to 40 to 60 μg/ml (depending on the compound), which could be substantially higher than the amount of compound that is naturally present in the crude extract. Thus, it is possible that the effect exhibited by the extract is a result of combinations of different compounds at lower concentrations than those tested. Also, differing compounds than those tested in our study could be also contributing for the extract's activity.

The results of this study demonstrated that the combination of extracts **E1**, **E7** and **E2** with doxorubicin showed a more potent an anticancer effect in A549 lung cancer cell line, with the increase of cell death mediated mechanisms when compared with the use of doxorubicin alone. Three compounds isolated from extract **E2** (**C2**, **C5** and **C7**) in combination with doxorubicin demonstrated to have a higher effect in the decrease of cell viability when opposed to the use of **E7** in combination with doxorubicin. The interest upon the implementation of drug combinations with our extracts and compounds in A549 was validated. Further studies must endorse the evaluation of potential cellular and molecular targets involved in the cell death mechanism here demonstrated. Also, combinations with other commonly used anticancer drugs should be thought to offer broader in depth views of the extracts and compounds' potential.

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Conflict of interest

There are no conflicts of interest to report.

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CHAPTER 4

CONCLUSIONS AND
FUTURE PERSPECTIVES

4.1. Conclusions and future perspectives

The first phase of this study aimed the assessment of the *in vitro* anticancer bioactivity of crude ethyl extracts obtained from marine and soil-derived fungi *Neosartorya tsunodae* KUFC 9213, *Neosartorya laciniosa* KUFC 7896 and *Neosartorya fischeri* KUFC 6344, in a panel of seven cancer cell lines, in particular, in non-small-cell lung carcinoma (A549), malignant melanoma (A375), breast adenocarcinoma (MCF-7), colon carcinoma (HT29, HCT116), hepatocellular carcinoma (HepG2) and glioblastoma (U251) cells.

The extracts of *N. laciniosa* and *N. fischeri* presented *in vitro* anticancer activity by decreasing cell proliferation, clonogenic potential and increasing both DNA damage and cell death associated events (nuclear condensation and morphological alterations) in breast adenocarcinoma, malignant melanoma and both colon carcinoma cell lines.

The second phase had as an objective to evaluate whether a combination of doxorubicin with the crude ethyl extracts obtained from *Neosartorya tsunodae* KUFC 9213, *Neosartorya siamensis* KUFA 0017, *Neosartorya laciniosa* KUFC 7896, *Aspergillus similanensis* KUFA 0013, *Neosartorya paulistensis* KUFC 7894, *Talaromyces trachsypermus* KUFC 0021 and *Neosartorya fischeri* KUFC 6344 as well as with eight compounds isolated from *Neosartorya siamensis* KUFA 0017 enhanced doxorubicin's effect on cell viability. The eight isolated compounds were, respectively, 2,4-dihydroxy-3-methylacetophenone (**C1**), nortryptoquivaline (**C2**), chevalone C (**C3**), tryptoquivaline H (**C4**), fiscalin A (**C5**), *epi*-fiscalin C (**C6**), *epi*-neofiscalin A (**C7**) and *epi*-fiscalin A (**C8**).

The enhancement of doxorubicin's anticancer activity was indeed achieved when used in combination with extracts from *N. tsunodae*, *N. siamensis*, *N. laciniosa* in the A549 lung cancer cell line. The combinations of doxorubicin and extracts demonstrated a significant decrease in cell viability, and an increase in DNA damage and in cell death associated events (nuclear condensation and morphological alterations).

The promising results obtained with the combination of doxorubicin and *N. siamensis* extract in the A549 cells encouraged the evaluation of the effect on cell viability of compounds **C1** to **C8**, in combination with doxorubicin in the same cell line. Although these compounds are not novel isolates, having also been isolated from other fungi of terrestrial origin, and concomitantly screened for anti-proliferative activity in a few cancer cell lines, to our knowledge, it is the first time that these compounds were evaluated in a combinatory regimen for the enhancement of the anticancer activity of doxorubicin in a lung cancer cell line. Results demonstrated that all (**C2** to **C8**) but one compound (**C1**) exhibited an

enhancement of the doxorubicin effect in the decrease of cell viability in A549. In particular, compounds **C2**, **C5** and **C7** displayed a more significant decrease in A549 cell viability than the exhibited by the use of extract from *N. siamensis* in combination with doxorubicin.

Moreover, the overall results obtained with this screening suggest that compounds **C2**, **C5** and **C7** may in fact be the compounds responsible for the anticancer effect observed with the *N. siamensis* extract in the A549 lung cancer cell line.

Future approaches that should be followed in the sequence of this study should involve the better understanding of the extent of the effectiveness of the combinatorial regimens of extracts and doxorubicin / compounds and doxorubicin.

In our view, there is particular interest in the isolation and anticancer screening, and in getting further mechanistic insights, of compounds from the extracts that presented significant enhancement of doxorubicin's activity, namely those derived from *N. tsunodae* and *N. laciniosa*. In particular for *N. tsunodae*, which demonstrated no cytotoxic effect when used alone, while in combination with doxorubicin demonstrated a high cytotoxic effect.

A greater range of extract/compound concentrations must be tested in order to calculate the combinatory index (CI), which will effectively determine whether the combination is of synergistic, additive or antagonistic nature.

The combination of doxorubicin and compounds must also be subjected to assessment of DNA damage and induction of cell death by apoptosis. The analysis of apoptotic involvement should include assays for several apoptotic features, for example, assessing mitochondrial membrane potential, measurement of caspase activity, DNA fragmentation, phosphatidylserine (PS) externalization and morphological alterations by electron microscopy. The analysis of cell cycle arrest should also be ensued.

Having identified the pathway of cell death, further studies should be pursued in order to understand the specific interaction between the extracts/compounds and doxorubicin, as for example, assessing the accumulation of doxorubicin in the cells and P-glycoprotein expression. More importantly, considering that lung cancer is highly resistant to chemotherapy, the expression of the lung resistance-related protein (LRP) should be evaluated.

Additionally, other combinations with other chemotherapeutic drugs should be evaluated, in an effort to find more potential combinatory enhancement. Drugs that are commonly used in the treatment of lung cancer are of critical interest. Other lung cancer cell lines should also be used, in order to unravel possible differentiation between distinct lung can-

cer genetic profiles. Inclusively, testing these combinations on multi-drug resistant (MDR) cell lines may also be a preferable path for getting new perspectives in further studies.

APPENDIX

EXPERIMENTAL PROTOCOLS

P1. CELL CULTURE

Principle

Cell culture is based on the maintenance of disassociated cells *in vitro*, and in the case of cancer cells, these cells may be developed from primary cultures obtained from tumours, and may proliferate indefinitely in culture as cell lines. Cultured cells must be grown in a favorable artificial environment with the monitoring of physicochemical (e.g. temperature, pH, O_2 and CO_2 tension) and physiological conditions (e.g., nutrients and growth factors). General cell maintenance also ensures that cell lines that grow in monolayer are sub-cultured into new flasks upon reaching 80-90% confluence by using enzymatic dissociation (e.g. trypsin). When an experimental assay is to take place, it is vital to ensure the continuity of the cell line by sub-culturing, and subsequently proceeding to the calculus of cell viability and density for the preparation of cell suspension at the desired density (Langdon, 2004; Freshney, 2010).

Materials	Assay solutions and reagents
T25 or T75 cell culture flasks	PBS (Phosphate Buffered Saline*)* buffer
Humidified CO2 incubator	0.25% Trypsin/EDTA solution
Inverted contrast phase microscope	Cell culture medium (MEM, DMEM and RPMI)
Laminar flow chamber	Fetal Bovine Serum (FBS)
Neubauer chamber	HEPES
	Sodium bicarbonate
	Sodium pyruvate
	Penicillin and Streptomycin (Antibiotics)

CELL CULTURE MAINTENANCE AND MANIPULATION [2]

Starting a new cell culture

1. When starting a fresh culture of cells, use an aliquot of cells preserved in a liquid nitrogen cell container. Prefer aliquots with a smaller passage number.
2. Quickly thaw the cells, placing all the content of the aliquot into a sterile culture flask, with 4 ml of cell culture medium at 37ºC. This procedure avoids toxicity from the DMSO in the freezing medium.
3. Shake the flask with care to evenly spread the cell solution.
4. Incubate the cells in a humidified incubator at 37ºC and 5% CO_2.
5. Do not move the flasks during the 24 h incubation period.
6. After adhered, change the medium to remove remains of DMSO.

Changing cell medium

1. Every two days, change the medium of cultured flasks.
2. Before starting the procedure, be sure to examine for confluence, dead cells and contamination under an inverted contrast phase microscope.
3. Heat PBS and cell culture medium to 37ºC in an appropriate and uncontaminated water bath.
4. In the laminar flow chamber, carefully remove the medium from the flask.
5. Wash the bottom of the flask with 1 ml of PBS to remove debris, this point may be repeated as desired. Remove the PBS.
6. Add 5 ml of appropriate cell culture medium.
7. Incubate cells in a humidified incubator at 37ºC and 5% CO_2.

[2] All protocols involving cell maintenance and manipulation must be done under sterile conditions. The use of a laminar flow chamber and aseptic techniques is essential.

Sub-culturing

1. Cells will generally need to be sub-cultured once a week or when a confluence > 80% is reached.
2. Heat a trypsin/EDTA solution to 37ºC in an appropriate and uncontaminated water bath. It is preferable to have the medium and PBS at room temperature.
3. Remove the medium from the flask with care.
4. Wash the cells twice with 1 ml of PBS and remove after washing.
5. Add 1 ml of trypsin/EDTA solution and incubate for 5 min at 37ºC.
6. Observed under an inverted contrast phase microscope for detached and rounded cells.
7. When cells become detached add 4 ml of medium and slowly resuspend. Note: Could be necessary to resuspend cells before adding the medium if the cells form large aggregates.
8. Remove a certain amount of cell suspension, in accordance with cell proliferation speed, e.g. sub-culturing ratio of 1:8.
9. Add medium up to 5 ml and spread the cell suspension evenly.
10. Incubate in a humidified incubator at 37ºC and 5% CO_2.

References

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P2. MTT ASSAY

Principle

The MTT colorimetric assay allows the evaluation of the effects of a test compound on cell viability and cell proliferation (Vega and Pugsley, 2011). This assay corresponds to a simple and quick way to assess the cytotoxic or preventive effect of a particular substance in a designated cell line. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) is a water soluble tetrazolium salt that in viable cells is converted by mitochondrial pathways to formazan crystals, which are water insoluble and can be quantified by optical density (O.D) using a microplate reader at a wavelength of 570 nm. The amount of formazan crystals will be directly proportional to the amount of viable cells (Plumb, 2004).

Materials	Assay solutions and reagents
96-multiwell culture plates	PBS (Phosphate Buffered Saline) buffer
Microplate reader (570 nm)	MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)
	Solubilization solution – DMSO:Ethanol (1:1)

Assay solution preparation

I. 1X PBS (Phosphate Buffered Saline) buffer

	Final concentration	Weight
NaCl	137.0 mM	8.0 g
KCl	2.7 mM	0.20 g
Na_2PO_4	10.0 mM	1.44 g
KH_2PO_4	2.0 mM	0.24 g

Weight all reagents and add 800 ml of ultrapure H_2O. Subsequently, adjust pH to 7.4 by using HCl or NaOH solution as necessary. After adjusting the pH, add H_2O to a final volume of 1 L. Sterilize the solution by autoclaving and store at 4ºC.

II. MTT stock solution (5 mg/ml)

	Final concentration	Weight
MTT	5 mg/ml	50 mg

Dissolve 50 mg of MTT in 10 ml of PBS. Dissolve by the use of an ultrasound bath. Solution is aliquot as desired and store at -20ºC until use.

III. Solubilization solution – DMSO: Ethanol (1:1)

To obtain 500 ml of solubilization solution, add 250 ml of DMSO and 250 ml of absolute ethanol. Use a fume hood to avoid contact with toxic vapors. Store the solution protected from light at room temperature.

Assay procedure

Cell metabolization of formazan crystals

1. Work under aseptic conditions by using a laminar flow hood.
2. Incubate cells in a 96-multiwell culture plate with a final volume of 100 µl/well and a cellular density appropriate for each cell line (according with experimental design). Leave cells to adhere for 24 hours at 37ºC in the incubator.
3. Apply the desired treatment (according to experimental design). At this time add 10 µl of MTT [3] (0.5 µg/ml) to the initial control (Ci). Incubate the plate for two hours at 37ºC and 5% CO_2.
4. After incubation, remove the medium from the Ci without removing the crystals, which should be adhered to the bottom of the well.
5. Place the culture plate in the incubator, and let incubate for the remaining 46 h.
6. At the end of the treatment, add 10 µl of MTT (0.5 µg/ml) per well and incubate for two hours at 37ºC and 5% CO_2 [4]. Afterwards, remove the medium.
7. Add 150 µl of the solubilization solution (DMSO:Ethanol) [5] to each well.
8. Shake the plate gently for 10 min until a homogenous solution is obtained.
9. Read the optical density of the solution at a wavelength of 570 nm in a microplate reader. If possible, just before reading gently shake the plate.
10. The percentage of cell proliferation and cellular inhibition must be calculated by equations 1 and 2, respectively.

[3] MTT is highly toxic and carcinogenic, it must be disposed to a toxic waste container and never be mixed with normal waste. Handle with care.

[4] Due to the photosensitivity of the formazan crystals, from this point forward protect the plate from light. The following points do not require the use of a laminar flow hood.

[5] DMSO is toxic and highly volatile. Use the fume hood and handle with care.

Calculation of cell proliferation and IC_{50}

The half maximal inhibitory concentration (IC_{50}) can be calculated using dose-response analysis performed in appropriate statistical software.

The percentage of cell proliferation may be calculated according to the equation 1 [6]:

(1) **% of cell proliferation** = $(OD_{sample} - OD_{t=0\ h}) / (OD_{t=48\ h} - OD_{t=0\ h}) \times 100$.

The percentage of cell inhibition may be calculated according to the equation 2:

(2) **% of cell inhibition** = 100 - % of cell proliferation.

References

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Sylvester, P. W. (2011). Optimization of the tetrazolium dye (MTT) colorimetric assay for cellular growth and viability *Drug Design and Discovery* (pp. 157-168): Springer.

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[6] OD corresponds to optical density

P3. NUCLEAR CONDENSATION

Principle

Apoptosis is characterized by several hallmarks such as cell shrinkage, retraction of pseudopods, rounding of the cell, reduction of cellular volume, nuclear fragmentation and chromatin condensation (Wong, 2011). Nuclear condensation is a feature that is common to both mitosis and cell death, however, chromatin condensation is much more intense in the latter case, and ultimately culminates in nuclear fragmentation. The observation of this phenomenon is possible after appropriate cell isolation and fixation, with the staining of nuclear DNA with an adequate fluorochrome and observation under a fluorescence microscope. DAPI (4',6-diamidino-2-phenylindole) is a fluorochrome that binds with the A-T regions of the DNA and is consequently commonly used as a stain when assessing nuclear condensation (Toné, Sugimoto, et al., 2007).

Materials	Assay solutions and reagents
96-multiwell culture plates	PBS (Phosphate Buffered Saline) buffer
Centrifuge	0.25% Trypsin/EDTA solution
Cytocentrifuge	DAPI staining solution (1 µg/ml)
Hydrophobic barrier pen	50% Glycerol solution
Poly-L-Lysine microscope slides	4% Paraformaldehyde (PFA) (w/v)
Fluorescence microscope	

Assay solution preparation

I. 1X PBS (Phosphate Buffered Saline*)* buffer

	Final concentration	Weight
NaCl	137.0 mM	8.0 g
KCl	2.7 mM	0.20 g
Na_2PO_4	10.0 mM	1.44 g
KH_2PO_4	2.0 mM	0.24 g

Weight all reagents and add 800 ml of ultrapure H_2O. Subsequently, adjust pH to 7.4 by using HCl or NaOH solution. After adjusting the pH, add H_2O to a final volume of 1 L. Sterilize the solution by autoclaving and store at 4ºC.

II. DAPI staining solution (1 µg/ml)

From a DAPI stock solution of 100 µg/ml, prepare a 1 µg/ml working solution in ultrapure H_2O. Protect from light and store at -20ºC.

III. 50% Glycerol solution (v/v)

To prepare 10 ml of glycerol solution, mix 5 ml of glycerol and 5 ml of PBS (1X).

IV. 4% Paraformaldehyde (PFA) (w/v)

	Final concentration	Weight
Paraformaldehyde (PFA)	4%	4 g

For a 100 ml solution, weight 4 g of PFA and add PBS (1X) until reaching 100 ml. Heat the solution in a water bath at 60ºC and stir frequently. This solution will take several hours until totally dis-

solved. Allow the solution to cool at room temperature and fix the pH to 7.4. Store at -20ºC until use [7].

Assay procedure

Sample preparation

1. Incubate cells in a 24-multiwell culture plate with a final volume of 1000 µl/well and a cellular density appropriate for each cell line (according to experimental design), e.g. $0.1x10^6$ cells/ml.
2. Leave cells to adhere for 24 hours in the incubator at 37ºC and 5% CO_2.
3. Apply the desired treatment and leave to incubate for 48 hours (according to experimental design).
4. After the end of the treatment, collect the medium from each well and place in a respectively labeled centrifuge tube.
5. Wash each well with 1 ml of PBS (1X) and collect to the respective centrifuge tube.
6. Trypsinize each well with 150 µl of warm trypsin/EDTA solution and incubate at 37ºC and 5% CO_2 for about 5 min.
7. Stop the trypsinization process in each well by adding 400 µl of the respective previously collected medium. Resuspend the cells carefully.
8. Wash each well with 1ml of PBS (1X) and add to the respective centrifuge tube.
9. Centrifuge the tubes for 10 min at 2000 rpm.
10. Save 500 µl of the solution, and discard the remaining supernatant without disrupting the pellet.
11. Add 3 ml of PBS (1X) to the reserved 500 µl, and carefully resuspend.
12. Centrifuge the falcons for 10 min at 2000 rpm.
13. Save 500 µl of the supernatant and discard the remaining.

[7] Keep the solution in a well-sealed recipient it releases toxic vapors when heated

14. Resuspend the pellet in the 500 µl of supernatant.
15. Add 2 ml of 4 % paraformaldehyde solution and leave to incubate for at least 15 minutes at 37ºC.
16. Add 4 ml of PBS (1X) to each sample.
17. Centrifuge again at 2000 rpm for 10 min and repeat the steps 13 and 14.
18. Collect the sample into a respectively marked tube.
19. Store the samples at 4ºC. Samples will remain stable under these conditions for several months.

Nuclear staining

1. Identify poly-L-Lysine slides and mount into the appropriate cytocentrifuge frames. Poly-L-Lysine coated slides are advisable to ensure that cells attach to the slide during the cytocentrifugation.
2. Resuspend each sample and pipette around 80 µl - 120 µl of cell suspension into the cytocentrifuge tube [8].
3. Centrifuge the slides in a cytocentrifuge for 5 min at 500 rpm.
4. Unmount the slides carefully, making sure that the cells are adhered to the slide. At this stage you can verify if the volume of cells used is appropriated.
5. Circle the adherent cells with a hydrophobic barrier pen, and leave to air dry for 10-20 min.
6. Wash 3 times with PBS (1X) for 5 min.
7. Incubate with DAPI (1 µg/ml) for 10 minutes in the dark [9].
8. Add 6 µl of 50% glycerol solution to each sample, and place a coverslip.
9. Place the samples at – 20ºC and protected from light. Samples can be preserved at this temperature for several months. Alternatively, observe the sample immediately by using a fluorescence microscope.

[8] The volume used must take in account cellular density. High density will make visualization difficult. Low density could make the quantification of 300 nuclei impractical.

[9] DAPI is carcinogenic. Handle with care.

Visualization and quantification

Observe samples in a fluorescence microscope and keep the room with the lowest light conditions as possible. Stained nuclei will appear with a blue coloration. The correct quantification of nuclear condensation requires a clear distinction between this event and other physical structures such as debris, damaged nuclei, among others that may resemble a result of nuclear condensation. The above-mentioned situations must never be quantified as nuclear condensation. The visualization of nuclear condensation does not appear as a standardized image, yet one must consider that an array of slightly altered situations will correspond to a positive count of nuclear condensation.

Quantification of nuclear condensation is obtained by counting at least 300 cells with condensed nuclei. The percentage of nuclear condensation is calculated by applying the following equation:

% of cells with condensed nuclei = (number of cells with condensed nuclei / total number of cells counted) x 100

At least 3 independent experiments must be done. It is advisable that the same researcher is responsible for all the visual quantification of the whole group of experiments, so that the same criteria will be maintained.

References

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P4. COMET ASSAY

Principle

The single cell gel electrophoresis assay or “comet assay” is a method for measuring DNA damage in single cells. The principle of the assay consists on the migration of the loops/fragments of DNA through an agarose gel when submitted to an electric field. This assay includes several steps, such as obtaining of isolated cells, mixing with agarose, cellular lysis, alkaline treatment (DNA unwinding), electrophoresis (DNA migration), staining with DNA-specific fluorescent dyes and microscope analysis. The alkaline version of the comet assay (the pH of the alkaline treatment is more than 13) allows the detection of DNA strand breaks and alkali labile sites. The presence of strand breaks relaxed supercoiled DNA allowing the migration of the DNA loops giving an image like a comet in sky. The use of a fluorescence microscope to capture and visualize and appropriate software for imaging analysis (e.g. CometScore®) will give information on the % of DNA damage through the % of DNA in the comet tail. DNA migration is directly proportional to DNA damage (Collins, 2004; Olive and Banáth, 2006; Collins, Oscoz et al., 2008).

Materials	Assay solutions and reagents
24-multiwell culture plates	PBS (Phosphate Buffered Saline*)* buffer
Horizontal electrophoresis tank	0.25% Trypsin/EDTA solution
Electric power supply	DAPI staining solution (1 µg/ml)
Microscope slides	1% Normal Melting Point Agarose (NMP) (w/v)
Fluorescence microscope	0.5% Low Melting Point Agarose (LMP) (w/v)
	Lysis solution, pH 10
	Electrophoresis Buffer
	Absolute ethanol

Assay solution preparation

I. 1X PBS (Phosphate Buffered Saline*)* buffer

	Final concentration	Weight
NaCl	137.0 mM	8.0 g
KCl	2.7 mM	0.20 g
Na_2PO_4	10.0 mM	1.44 g
KH_2PO_4	2.0 mM	0.24 g

Weight all reagents and add 800 ml of ultrapure H_2O. Subsequently, adjust pH to 7.4 by using HCl or NaOH solution. After adjusting the pH, add H_2O to a final volume of 1 L. Sterilize the solution by autoclaving and store at 4ºC.

II. 1% Normal Melting Point Agarose (NMP) (w/v)

Prepare a solution with 200 mg of normal melting point agarose (NMP) and distilled water until 20 ml. To dissolve the solution, heat in a microwave at full power for 1 to 2 min. Store the solution at 4ºC. When required, melt the solution before use.

III. 0.5% Low Melting Point Agarose (LMP) (w/v)

Prepare a solution with 100 mg of low melting point agarose (NMP) and PBS (1X) until 20 ml. To dissolve the solution, heat in a microwave at full power for 1 to 2 min. Store the solution in 2 ml aliquots at 4ºC. When required, melt the solution before use.

IV. Lysis solution, pH 10

	Final concentration	Weight
NaCl	2.5 M	146.1 g
Na_2EDTA	100.0 mM	37.2 g
Tris Base	10.0 mM	1.21 g
NaOH to pH of 10		Approx. 7 g

For 1 L of solution add 146.1 g of NaCl, 37.22 g of Na_2EDTA, 1.211 g of Tris Base and around 5 g of NaOH (save ~2 g for further use). Add distilled water until reaching the 900 ml and stir the solution. The remaining 2 g will have to be gradually added to the solution as the dissolution occurs and until reaching the correct pH. When totally dissolved add the remaining volume of distilled water up to a volume of 1 ml. Store the solution at 4ºC. Add triton X-100 1% (v/v) immediately prior to use.

V. Electrophoresis Buffer (300 mM NaOH, 1 mM Na_2EDTA, pH 13)

	Final concentration	Weight
NaOH	3 M	120 g

Add NaOH and distilled water until reaching 1 L. Dissolve and store at 4ºC.

Na_2EDTA	200 mM	3.722 g

Add distilled water until reaching 50 ml. Dissolve and store at 4ºC.

Mix only both solutions immediately prior to use. For 1 L of electrophoresis buffer use 100 ml of 3M NaOH, 5 ml of 200 mM Na_2EDTA and 895 ml of distilled water. The pH of this solution should be ≥ 13. Wear protective gloves and handle with care.

VI. DAPI staining solution (1 µg/ml)

From a DAPI stock solution of 100 µg/ml, prepare a 1 µg/ml working solution in ultrapure H_2O. Protect from light and store at -20ºC.

Assay procedure

The elaboration of this protocol will require an anticipated preparation of the material and reagents mentioned in the "Assay solutions and reagents/Materials" section. A thoughtful reading of this section is mandatory.

Slide preparation

Coat one side of a conventional microscope slide dipping the slide in melted 1% NMP agarose (w/v) solution. Place horizontally and leave to air dry at room temperature for 10 min. Then place the slides overnight at 37ºC to dry completely.

Cell preparation

1. Incubate cells in a 24 multi-well plate with a final volume of 1000 µl/well and a cellular density appropriate for each cell line (according to the desired experimental design), e.g. $0.1x10^6$ cells/ml. Leave cells to adhere for 24 hours in the incubator at 37ºC and 5% CO_2.
2. Apply the desired treatment and expose cells for 4, 24 and 48 hours (according to the experimental design).
3. After reaching the end of the treatment, remove the medium from each well and wash twice with 200 µl of PBS (1X).

4. Trypsinize each well with 100 µl of trypsin/EDTA solution and incubate at 37ºC and 5% CO_2 for about 5 min.
5. Stop the trypsin action by adding 400 µl of cold medium to each well and resuspend.
6. Count the cells and prepare a cell suspension ($5x10^4$ cells/ml) for each sample. From here on, keep the cells on ice to prevent the activation of DNA damage repair mechanisms.
7. Centrifuge cells for 1 min at 5,000 rpm. If the volume of cells is lower than 10 µl, this step is unnecessary.
8. Remove the supernatant, and add 100 µl of LMP agarose and resuspend each sample. LMP agarose must be melted before using (melting point ~60ºC) be sure to cool down to 37ºC in a water bath as to not damage the cells.
9. Apply ~75 µl of the cell suspension (cells plus LMP agarose) to the slide and place a coverslip over it. One slide is suitable for two samples. Keep the slides at 4ºC around 5 to 10 min.
10. When the LMP agarose in the slides is solidified (~ 10 min at 4ºC), remove the coverslips with care, to avoid damaging the gel.
11. Fully immerse the slides in the lysis solution.
12. Incubate for 2 hours at 4ºC in the dark. Possible stop point - if necessary leave overnight.
13. Pour the electrophoresis buffer solution into the electrophoresis tank and maintain at 4ºC.
14. After incubation, rinse the slides with distilled water and place them in the electrophoresis tank.
15. Incubate the slides for 40 minutes in electrophoresis buffer at 4ºC to unwind the DNA. Place the label in the direction of the anode.
16. Run the electrophoresis at 20 V (1V/cm) for 20 minutes at 4ºC.
17. Remove slides from the tank and wash 3 times in distilled water for 5 minutes each. Be careful not to damage the gel on the slides.
18. Dehydrate the slides by immersing them twice for 5 minutes in absolute ethanol. Leave to air dry.
19. Stain each sample with 20 µl of DAPI (1 µg/ml).

20. Samples may now be observed under a fluorescence microscope.

Evaluation of DNA damage

DNA damage evaluation is assessed by fluorescence microscopy using a 20X or a 40X objective. Avoid cells in the gel borders and make sure that you are not capturing the same area repeatedly.

Capture at least 100 cells per sample, and process the image by using comet analysis software, such as CometScore®. Express the results as percentage of DNA in the tail, additional results may be expressed as tail moment or tail length.

References

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P5. CLONOGENIC ASSAY

Principle

The clonogenic or colony forming assay is a cell survival assay based which is used to analyse indefinite cell proliferation. This assay determines whether a single cell previously exposed to a desired toxic agent maintains a long-term ability to form a colony. Results obtained from this experiment allow the understanding of whether the toxic agent used possesses cytostatic activity by altering the cell's reproductive viability (Franken, Rodermond et al. 2006; Haloom, Christian et al. 2011).

Materials	Assay solutions and reagents
24-multiwell culture plates	PBS (Phosphate Buffered Saline*)* buffer
12-multiwell culture plates	0.25% Trypsin/EDTA solution
Stereomicroscope	4% Paraformaldehyde (PFA) (w/v)
Laminar flow hood	0.05% Crystal violet (w/v)

Assay solution preparation

I. 1X PBS (Phosphate Buffered Saline*)* buffer

	Final concentration	Weight
NaCl	137.0 mM	8 .00g
KCl	2.7 mM	0.20 g
Na_2PO_4	10.0 mM	1.44 g
KH_2PO_4	2.0 mM	0.24 g

Weight all reagents and add 800 ml of ultrapure H_2O. Subsequently, adjust pH to 7.4 by using HCl or NaOH solution. After adjusting the pH, add H_2O to a final volume of 1 L. Sterilize the solution by autoclaving and store at 4ºC.

II. 4% Paraformaldehyde (w/v)

	Final concentration	Weight
Paraformaldehyde (PFA)	4%	4 g

For a 100 ml solution, weigh 4 g of PFA and add PBS (1X) until reaching 100 ml. Heat a water bath at 60ºC and stir it frequently, this solution will take several hours until become total dissolved (keep the solution in a well-sealed recipient because it releases toxic vapors when heated). Wait until it settles at room temperature and fix pH=7.4. Store at -20ºC until use.

III. 0.05% Crystal violet (w/v)

	Final concentration	Weight
Crystal violet	0.05%	5 mg

Add 5 mg of crystal violet and add up to 100 ml with PBS (1X).

Assay procedure

Cell preparation

1. Incubate cells in a 24 multi-well plate with a final volume of 1000 µl/well and a cellular density appropriate for each cell line (according to the desired experimental design), e.g. $0.1x10^6$ cells/ml.
2. Leave cells to adhere for 24 hours in the incubator at 37ºC and 5% CO_2.
3. Apply the desired treatment and expose cells for 48 hours (according to the experimental design).
4. After reaching the end of the treatment, remove the medium from each well and wash twice with 200 µl of PBS (1X).
5. Trypsinize each well with 100 µl of trypsin/EDTA solution and incubate at 37ºC and 5% CO_2 for about 5 min.
6. Stop the trypsin action by adding 400 µl of cold medium to each well and resuspend.
7. Count the cells and prepare serial dilutions to prepare a final cell suspension of 200 cells in 1 ml for each sample. Resuspend every dilution with care, to ensure that the final solution contains the right amount of cells.
8. Plate the cell suspension of 1 ml containing 200 cells in a 12-multiwell culture plate.
9. Leave cells to incubate for 10 days in the humidified incubator at 37ºC and 5% CO_2.

10. At the end of the incubation period, remove the medium and was with 2 ml of warm PBS.
11. Cells must then be fixed in situ with 2 ml of 4% paraformaldehyde and left to incubate for 15 min at 37ºC.
12. Remove the paraformaldehyde and wash with 2 ml of warm PBS.
13. Remove the PBS and add 2 ml of 0.05% crystal violet dye, and leave to incubate for 30 min at room temperature.
14. Remove the crystal violet and wash with distilled water. Be careful as to not lift the cells.
15. Leave to air-dry overnight.
16. The plate is now ready to be examined. Under a stereomicroscope, count cell colonies with more than 50 cells.

Calculations

With the data obtained it is possible to calculate the plating efficiency through the ratio of the number of colonies with more than 50 cells and the number of cells plated. As a consequence, the surviving fraction in relation to the negative control is calculated according to the following equation:

$$\textbf{\% Surviving fraction (SF)} = (PE_{\text{treated cells}} / PE_{\text{control}}) \times 100$$

References

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